

Tumour hypoxia

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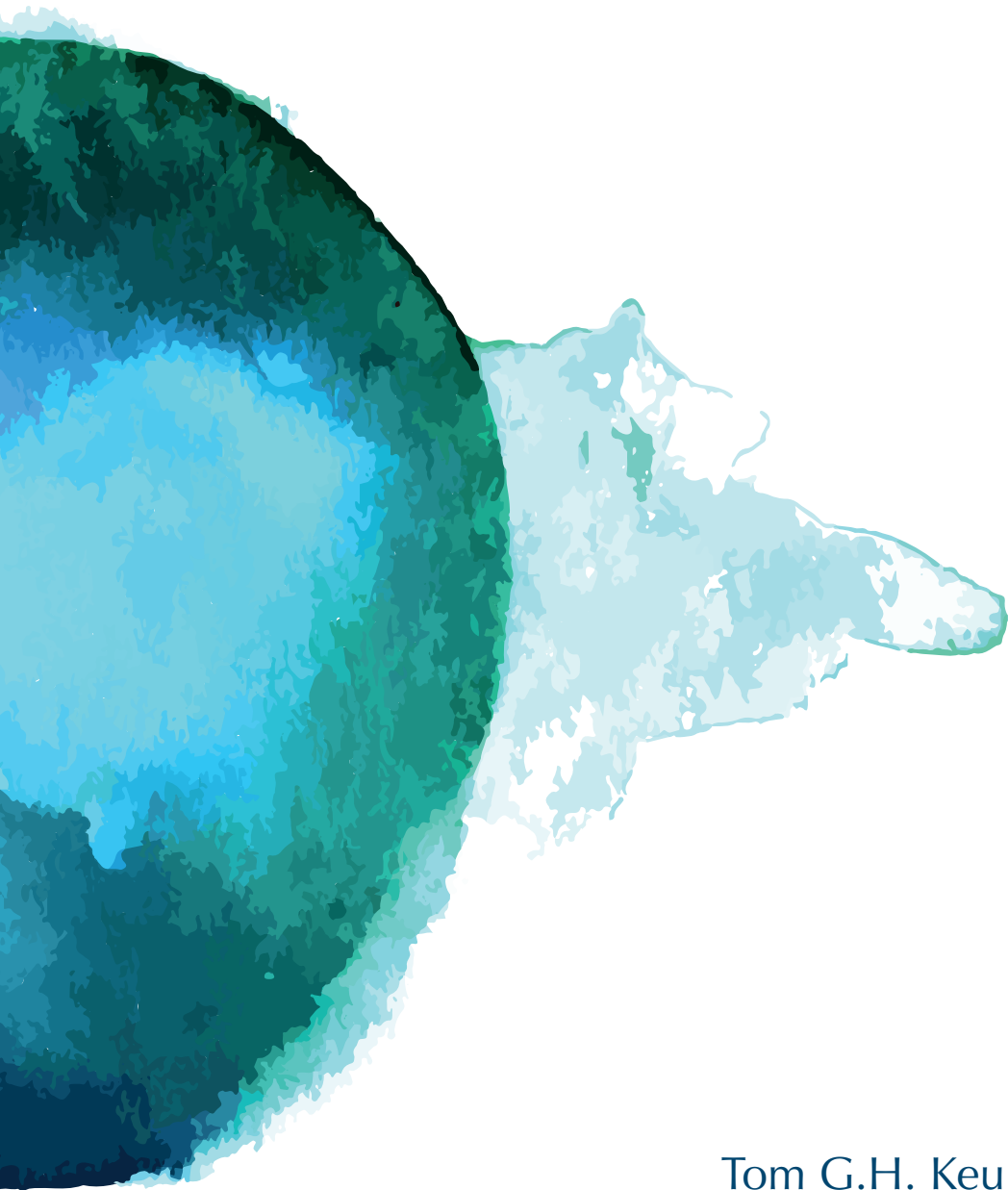
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Tumour hypoxia effects on autophagy and intercellular communication



Tom G.H. Keulers

Tumour hypoxia

Effects on autophagy and intercellular
communication

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Tumour hypoxia

Effects on autophagy and intercellular communication

DISSERTATION

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in accordance with the decision of the Board of Deans,
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by

Tom Gertrudis Hubertus Keulers

Supervisors

Dr. K.M.A. Rouschop

Prof. dr. M.A.G.G Vooijs

Assessment Committee

Prof. dr. F.C.S. Ramaekers (Voorzitter)

Dr. E.N.M. Nolte-'t Hoen (Utrecht University)

Prof. dr. R.M. Schiffelers (Utrecht University)

Prof. dr. P. Schrauwen

Dr. L. Wieten

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Chapter 1

Introduction

Introduction

Cancer is a devastating disease that 1 in 3 people in their lifetime are faced with. In Europe, 3.7 million new cases and 1.9 million deaths are reported annually, which makes cancer the second most important cause of death and morbidity (1). Due to the aging of our population this incidence will increase in the coming decades. Although many advances in treatment of cancer have been made, novel therapeutic strategies and further understanding of the complexity of the disease and mechanisms of disease progression are highly needed to further increase the therapeutic effectiveness and cure of patients.

Cancer originates from cells that have lost the control over cell proliferation and cell death. Solid tumours evolve into complex tissues that contain, in addition to cancer cells, other recruited cell types including cells of the immune system, the tumour blood- and lymphatic vasculature and fibroblasts. Together with extracellular matrix this creates the tumour microenvironment (TME). Cells of cancerous and non-cancerous origin in the TME constantly interact and thereby contribute to development of the tumour. The TME of solid tumours is characterized by poorly oxygenated regions (hypoxia) and regions that receive limited nutrients. Hypoxia occurs when oxygen demand exceeds delivery due to alterations of the metabolic and proliferative status of tumour cells or as a result of insufficient blood delivery due to an immature and chaotic tumour vasculature. In general, two distinct patterns of hypoxia are considered. 1) Diffusion-limited, or chronic, hypoxia which is caused by limitations in oxygen diffusion from the tumour vasculature in the surrounding tissue or increased O_2 consumption rates. Typically, cellular proliferation results in increased distance of cells to the nearest perfused vessel. Oxygen consumption by cells closer to the vessel results in reduced delivery and creates oxygen gradients in relation to distance from the vessel. During diffusion-limited or chronic hypoxia, oxygen levels vary at a relatively slow rate (hours to days). 2) Perfusion-limited, or acute hypoxia is caused by temporal occlusion of vessels and leads to rapid fluctuations between oxygenation (minutes to hours)(2). Typically, normal tissues vary between 3.9% and 6.8% O_2 (3). In cancer this is much more diverse. For example in breast (4), cervix (5), head and neck (6) and prostate carcinomas (7), oxygen levels vary between normal oxygenation to complete anoxia.

Tumour hypoxia is a driver for selection of cells that have lost their apoptotic potential, as firstly described by Greaber et al. (8) who showed that p53 deficient cells take over the population under the pressure of hypoxia. From a clinical point of view, means of reducing the hypoxic fraction of tumours is highly desired since low oxygenation of tumours is associated with poor outcome in multiple cancer types, independent of treatment modality (9,10). In HNSCC, where a meta-analysis indicated that the degree of hypoxia is the most significant factor explaining variability in survival (11), the evidence is strongest. The observed effect on local control is most likely caused by in sensitivity of hypoxic cells to both chemo- and radiotherapy. Additionally there is an associa-

tion between hypoxia and the occurrence of metastasis (12). It has been proposed that tumour hypoxia contributes to malignancy e.g. through activation of epithelial to mesenchymal transition (13-16), stimulation of blood vessel growth and formation of the pre-metastatic niche through secretion of microvesicles/exosomes/extracellular vesicles (hereafter indicated by EV) (17-25). Importantly, in head and neck squamous cell carcinoma (HNSCC) evidence of the therapeutic benefit of hypoxia modification is strongest (26), indicating that hypoxia modification does not only influence local control, but also overall outcome.

In short, tumour hypoxia leads to the expansion of cells with a more invasive, metastatic and therapy resistant phenotype. Clinical studies show that hypoxia contributes to poor clinical outcome as it decreases loco-regional control, decreases disease free-survival and overall survival in head and neck squamous cell carcinoma (HNSCC) (27) (28). Therefore it is essential to understand hypoxia and associated processes in order to selectively target these areas.

Hypoxia responsive pathways

In response to hypoxia, cells activate oxygen sensitive pathways to adapt and survive these severe conditions. The hypoxia inducible factor (HIF) pathway is the most investigated hypoxia-responsive pathway. HIF are heterodimers composed of an oxygen-regulated subunit (HIF1 α , 2 α and 3 α , collectively referred to as HIF α) and a β -subunit (HIF1 β /ARNT1, ARNT2 and ARNT3). When oxygen is present, HIF1 α undergoes enzymatic hydroxylation by the O₂ regulated prolyl-4-hydroxylases (PHDs) and will be degraded by the proteasome after binding by the von Hippel-Lindau (pVHL) tumour suppressor. In contrast, during hypoxia, PHD enzymes are inhibited and HIF1 α hydroxylation leads to HIF1 α stabilization leading to transcriptional activation of target genes after binding to hypoxia responsive elements (HRE) in the promoter regions. HIF activation is associated with enhanced angiogenesis by secretion of growth factors such as VEGF, altered metabolism by the regulation of glycolytic genes to promote glycolysis, migration by activating the Wnt/ β -catenin or PI3K/AKT pathway, invasion and metastasis (reviewed in (29)).

During severe hypoxia (<0.2%O₂), the folding of proteins in the endoplasmic reticulum (ER) is compromised and misfolded proteins accumulate in the ER (30). As a result, the unfolded protein Response (UPR) is activated. Activation of the UPR is initiated by three ER-transmembrane stress sensors: Activating Transcription Factor 6 (ATF6), PKR)-like endoplasmic reticulum kinase (PERK) and Serine/threonine-protein kinase/endoribonuclease (IRE1). Typically these sensors are kept in an inactivated state through binding to heat shock protein family A (Hsp70) member 5 (HSPA5/ also known as BiP/GRP78). Due to the higher affinity of BiP to unfolded proteins, accumulation of unfolded proteins results in BiP-dissociation from the sensor and thereby activating them. Activation of the UPR leads to decreased overall protein synthesis, translation of specific proteins, increased protein traf-

ficking through the ER and increased protein degradation by ER-associated degradation (ERAD) and autophagy.

Autophagy is a cellular degradation- and recycle mechanism to maintain homeostasis of the cell and achieves this by recycling damaged and aged cytoplasmic content, such as aggregated or misfolded proteins and organelles. In mice, autophagy enables survival to neonatal starvation by preventing energy depletion (31). Furthermore, defective autophagy in brain causes accumulation of damaged mitochondria and protein aggregates and finally neuronal degeneration (32,33). Identically, in the liver autophagy-defects cause protein accumulation, hepatocyte cell death and liver failure (34). These findings support the pro-survival role for autophagy by maintaining energy homeostasis and removal of toxic products and damaged organelles (35).

Autophagy is continually active at a basal level, however during stress, like hypoxia or nutrient deprivation, autophagy is induced. Previously we showed that ATF4 (transcription factor downstream of the PERK) activates transcription of the autophagy-associated proteins, including microtubule-associated protein light chain 3 beta (LC3B) and autophagy related-5 (ATG5) (36) and GABARAPL1 (this thesis, **chapter 3**). In line, in tumours autophagy is mainly localized in hypoxic regions of tumours, sustaining the presence of hypoxic tumour regions (37-40), a feature associated with tumour progression (41).

During autophagy, cytoplasmic content is recognized by substrate specific adaptor proteins that recruit LC3B to the site where the initial membrane, or phagophore, will be generated. LC3B is generated as a precursor protein which is immediately cleaved by autophagy related 4 cysteine peptidase (ATG4) to expose a C-terminal glycine for further modification. This cytosolic form of LC3B is also referred to as LC3B-I. LC3B-I is then conjugated to phosphatidylethanolamine (PE) by an ubiquitination-like conjugation system where ATG7 acts as an ubiquitin-activating enzyme (E1). LC3B-I is transferred to the conjugating enzyme ATG3 (E2) where after it is conjugated to PE, forming the membrane-bound LC3B-II that coats the inner and outer membrane of the double-membrane, which allows the phagophore to grow. Efficient LC3B-PE formation requires the ATG12-ATG5-ATG16(L) complex. For their interaction, ATG12 is activated by the E1-activating enzyme ATG7. Thereafter, ATG12 is transferred to ATG10 (E2-conjugating enzyme) and conjugated to ATG5. ATG5 interacts with ATG16(L) to form the complex, which can form a tetramer via ATG16 homodimerization (reviewed (42)). The complex fulfills multiple functions such as determination of the site for LC3/GABARAP family protein conjugation to PE. The phagophore expands until the content is completely enclosed by the autophagic vesicle, referred to as the autophagosome. Eventually, autophagosomes fuse with hydrolase and protease-containing lysosomes to degrade the content of the autophagosome, which is then effluxed into the cytosol, as amino acids, monosaccharides, fatty acids and nucleotides that are used to maintain biosynthesis and energy homeostasis of the cell.

LC3B turnover is used as a marker of autophagic structures and is used as a “golden standard” in assessing autophagy activation and rate of autophagy (autophagic flux) (43).

LC3B-II is localized on the intraluminal and cytoplasmic site and autophagosomes. After autophagosome/ lysosome fusion, cytoplasmic LC3B-II is delipidated by ATG4 and recycled. Intraluminal LC3B-II is degraded by after lysosomal hydrolases during autolysosome formation. Therefore, LC3B-II turnover can be used to monitor autophagic activity (44).

Autophagy is considered a doubled-edged sword. In normal cells autophagy has a cytoprotective role by degrading cytotoxic components, and thereby prevents disease. In the tumour microenvironment, autophagy plays a similar role. By activating autophagy, cells survive nutrient depriving conditions, driving hypoxic tumour cells into a more aggressive, therapy resistant and metastatic phenotype. However, the stress tumour cells encounter (hypoxia, nutrient deprivation) are greater, making tumour cells more dependent on autophagy.

The LC3/ GABARAP protein family

The LC3/GABARAP protein family consists of the LC3 and GABARAP subfamilies and are characterized by high degrees of homology. LC3B is the most studied and characterized member of the protein family and is required for autophagosome formation. The LC3/ GABARAP protein family consisting of microtubule associated protein 1 light chain 3 alpha (LC3A) (2 splice variants), LC3B, microtubule associated protein 1 light chain 3 gamma (LC3C)), and GABA(A) receptor-associated protein (GABARAP), GABA(A) receptor associated protein Like 1 (GABARAPL1) and GABA(A) receptor associated protein Like 2 (GABARAPL2) are implicated in autophagy. The LC3/ GABARAP protein family members share a high degree of homology, though they are unique in function and implicated in autophagy-independent mechanisms as well. An extensive review about autophagy (un-) related functions of all LC3/ GABARAP protein family members is presented in **chapter 2**.

Initially, the GABARAP protein family members have been implicated in intracellular trafficking and transport of receptors to the plasma membrane (45-48). For example, GABA (A) receptor associated protein GABARAP derives its name from transport of the GABA(A) receptor (GABAAR) to the plasma membrane. Further research revealed that surface expression of other receptors like the angiotensin II (49), the epidermal growth factor receptor (EGFR) (50) and the human kappa opioid receptor is also mediated by GABARAP family proteins (48). However, retrograde transport is not limited to the transport of receptors alone. The GABARAP protein family, and especially GABARAPL1, is also implicated in (unconventional) secretion and the secretion of extracellular vesicles (**chapter 7 and 8**). A comprehensive review about autophagy related proteins and secretion is presented in **chapter 6**

Extracellular vesicles

From yeast to mammals, cells secrete extracellular vesicles (EVs) as a vehicle of intercellular communication. EVs are phospholipid bilayer enclosed vesicles that contain bioactive molecules such as nucleic acids (DNA, RNAs, miRNAs), lipids and proteins (receptors, enzymes, transcription factors and extracellular matrix proteins). EV is a generic name for vesicles originating from various cellular compartments including exosomes and microvesicles, but also apoptotic bodies are considered EVs. EVs are classified on their origin: Exosomes, as firstly described by Johnstone et al. (51), are generated during the maturation of early into late endosomes. During this process, intraluminal vesicles (ILVs) are formed by inward budding of the endosomal membrane forming multivesicular bodies (MVB) (52). MVBs can either fuse with lysosomes for degradation or fuse with the plasma membrane, resulting in release of the ILVs (exosomes) into the extracellular environment. Microvesicles are a different class of EVs and are released directly by budding from the plasma membrane (53). Although there are no markers to distinguish exosomes and microvesicles after their release, they are all enriched in tetraspanins such as CD9, CD81, and CD63 (53).

In addition to their role in normal physiology, such as their role in T-cell activation (54), EVs are implicated in several steps that stimulate cancer progression and cover most of the hallmarks of cancer described by Hannahan and Weinberg (55-57), including angiogenesis (18,58), increased proliferation(59), increased migration and invasion (60), metastasis (61), immune evasion (62-64), pre-metastatic niche formation (61,65) and inflammation (66).

For example, to meet the increasing demand of oxygen and nutrients of the tumour, blood vessel formation (angiogenesis) is essential. Several studies have shown that EVs contain pro-angiogenic cargo like pro-angiogenic mRNA's (67-69) and growth factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (70)(71). GBM tumour cells release pro-angiogenic EVs that are taken up by microvascular endothelial cells in the brain promoting tumour growth and stimulate tumour cell proliferation at distant sites (72). Another study performed with GBM cells, demonstrated that EVs derived from hypoxic GBM cells elicit pro-angiogenic responses in HUVEC cells *in vitro* and stimulate angiogenesis by inducing cytokine and growth factor secretion and thereby promote pericyte migration (73). Furthermore, EVs are shown to contribute to formation of the pre-metastatic niche (65,74). During the metastatic cascade, tumour cells detach from the primary tumour and disseminate throughout the body. Via the circulatory system circulating tumour cells invade the distant tissue which provides a suitable microenvironment for colonization. Studies have shown that suitable microenvironments can be formed before the arrival of circulating tumour cells (75). EVs contribute to the formation of these predetermined regions, also referred to as pre-metastatic niches (75,76), for instance by destroying the vascular barrier though induction of vascular leakiness (77), extracellular matrix remodelling (78) or

reprogram bone marrow progenitors cells towards a pro-vasculogenic phenotype (79). Taken together, EVs are involved in a wide variety of cancer associated processes contributing to tumour growth.

Scope of this thesis

The scope of this thesis is to gain insight in autophagy-associated proteins and their contribution to tumour development with particular focus on GABARAPL1, a member of the LC3/ GABARAP protein family; a group of proteins mainly involved in the recycle and degradation mechanism autophagy. The LC3/ GABARAP protein family is characterized by high degree of structural homology, suggesting that LC3/GABARAP family proteins exert similar functions. In **chapter 2** we aimed to clarify the functional differences of the LC3/GABARAP protein family members in autophagy (un)-related functions based on available literature. In **chapter 3** we demonstrate that the expression of the LC3/ GABARAP protein family member GABARAPL1 is induced during hypoxia in several cancer cell lines in a PERK-dependent manner. Additionally we show that GABARAPL1 is required for increased membrane expression of the epidermal growth factor receptor (EGFR) during hypoxia. EGFR is often mutated or amplified in cancer and has a significant impact on tumour development. In **chapter 4** we elaborate on the autophagy-dependency of EGFRvIII⁺ cells during stress conditions and the effect of chloroquine treatment on patients with EGFRvIII expressing tumours. In **chapter 5** we describe our findings that ATG12 expression is lost in a subset of HNSC patients. Further we investigated the effects of the lost ATG12 expression on tumour growth, therapy efficacy and tumour control

Autophagy is not exclusively required for the degradation of cellular content, but also mediates unconventional forms of secretion. In **chapter 6** we discuss the current knowledge of the physiological roles of autophagy-dependent secretion and the effects on the tumour microenvironment. Also autophagy-dependent receptor trafficking and its involvement in tumour development will be discussed. Further research on GABARAPL1 revealed that GABARAPL1 is required for the release of a subset of pro-angiogenic extracellular vesicles released during hypoxia (**chapter 7**). This subset is marked by GABARAPL1 expression on the surface of the vesicle, what makes them accessible for targeting or detection purposes. In this regard, GABARAPL1⁺ EVs are detectable and elevated in the blood of cancer patients. Furthermore, *in vitro* tests demonstrated that GABARAPL1⁺ EVs are targetable by antibodies, opening the window for biomarker or therapeutic development. EVs can contribute to metastasis formation by preparing the pre-metastatic niche. Therefore we explored the role of GABARAPL1⁺ EV in metastasis formation (**chapter 8**). Taken together, we show that autophagy is implicated in key cellular mechanisms associated with cell survival and communication in the tumour micro-environment.

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Chapter 2

LC3/GABARAP family proteins: Autophagy (un)related functions

Marco BE Schaaf*, Tom G Keulers*, Marc A Vooijs, Kasper MA Rouschop

Department of Radiation Oncology (Maastrro Lab), GROW School for Oncology &
Developmental Biology, Maastricht University Medical Center+, Maastricht,
The Netherlands.

* Authors contributed equally

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Abstract

From yeast to mammals, autophagy is an important mechanism to sustain cellular homeostasis through facilitating the degradation and recycling of aged and cytotoxic components. During autophagy, cargo is captured in double membraned vesicles, the autophagosomes, and degraded through lysosomal fusion. In yeast, autophagy initiation, cargo recognition, cargo engulfment and vesicle closure is Atg8 dependent. In higher eukaryotes, Atg8 has evolved into the LC3/GABARAP protein family consisting of 7 family proteins (LC3A (two splice variants), LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2). LC3B, the most studied family protein, is associated with autophagosome development and maturation, and is used to monitor autophagic activity. Given the high homology, the other LC3/GABARAP family proteins are often presumed to fulfill similar functions. Nevertheless, substantial evidence shows that the LC3/GABARAP family proteins are unique in function and important in autophagy-independent mechanisms.

In this review we discuss the current knowledge and function(s) of the LC3/GABARAP family proteins. We focus on processing of the individual family proteins and their role in autophagy initiation, cargo recognition, vesicle closure and trafficking, a complex and tightly regulated process that requires selective presentation and recruitment of these family proteins. In addition, autophagy unrelated functions of the LC3/GABARAP protein family members are discussed.

Keywords: autophagy, cancer, ATG7, Atg8 orthologues, LC3/GABARAP family proteins

Introduction

Autophagy (from the Greek auto-, "self" and phagein, "to eat") is a catabolic process required to maintain cellular homeostasis through directing old/damaged organelles (e.g. mitochondria), protein aggregates and pathogens for lysosomal degradation. The products of lysosomal breakdown (e.g. amino acids, fatty acids) are recycled and transported across the lysosomal membrane into the cytoplasm. These metabolites can be re-used in protein and ATP production (reviewed in (1)). During metabolic stress or periods of insufficient nutrient supply, autophagy is rapidly upregulated to sustain energy production and provide building blocks for essential cellular functions. In line, cells deprived of glucose or amino acids, or exposed to hypoxia depend on autophagy for their survival (2,3). In short, autophagy acts as a pro-survival mechanism through maintaining energy homeostasis and removal of toxic protein aggregates, pathogens and old/damaged organelles (4). Hence, defective autophagy in brain causes accumulation of damaged mitochondria and protein aggregates leading to neuronal degeneration (5). Similarly, autophagy-defects result in protein accumulation in the liver, causing hepatomegaly, hepatocyte cell death and liver failure (6).

Initiation of autophagy commences with the formation of an initial membrane structure, the phagophore (nucleation). As the phagophore elongates, it (non-)selectively sequesters cargo and closes into a double-membrane vesicle, the autophagosome. The autophagosome then fuses with a lysosome and exposes cargo to the lysosomal enzymes causing degradation of the content, a process well-conserved from yeast to humans. Research in yeast has provided insights into the role of essential proteins in autophagy. This has led to the identification of at least 38 AuTophagy-related proteins (Atg), of which many are functionally conserved in eukaryotes. In yeast, Atg8 is a limiting protein in expansion of the phagophore (7). Atg8 levels correspond with autophagosome size, as reduced Atg8 leads to the development of smaller autophagosomes with less favorable volume-to-surface ratios and attenuation of autophagy (7). In addition, Atg8 participates in cargo recruitment to the autophagosome by interacting with cargo binding proteins (e.g. Atg19) (8). For activation, Atg8 requires the covalent conjugation to phosphatidylethanolamine (PE) that enables its anchoring into the autophagosomal membranes, a process tightly orchestrated by Atg4, Atg7, Atg3 and the Atg12-Atg5-Atg16L1 complex (9).

In mammals, at least seven Atg8 orthologues exist; microtubule associated protein 1 light chain 3 alpha MAP1LC3A (two splicing variants hereafter referred as LC3A-a and LC3A-b), -beta (MAP1LC3B (LC3B), -gamma (MAP1LC3C (LC3C), GABA type A receptor-associated protein (GABARAP), GABARAP Like 1 (GABARAPL1/gec1) and GABARAP Like 2 (GABARAPL2/GATE16). The most studied and best understood LC3/GABARAP family protein is LC3B. LC3B is essential for the execution of autophagy and therefore the widely accepted marker for autophagy activity assessment. The LC3/GABARAP family proteins are produced from separate transcripts, derived from their own genomic loca-

tion (table 1). Nevertheless, they share a high sequence similarity and have a conserved carboxy-terminal glycine. In comparison to LC3B, LC3A-a, LC3A-b, LC3C, GABARAP, GABARAPL1 and GABARAPL2 display 92%, 94%, 71%, 59%, 60% and 65% similarity, respectively. Each LC3/GABARAP family protein is characterized by two amino-terminal α -helices and a carboxy-terminal ubiquitin core (10). LC3/GABARAP family proteins are often presumed to fulfil similar functions as LC3B. Functional differences and autophagy independent functions are frequently neglected and may lead to incomplete interpretation of data. Within this review we have provided an overview of the current knowledge on the function of the LC3/GABARAP family proteins.

We have attempted to provide information on several questions raised: e.g. a) Are their functions limited to autophagy? b) Are they all crucial for the execution of autophagy? c) What is their role in this process? d) Do they contribute to recruitment of selective targets for autophagy? If so, how is selectivity and recruitment organized? e) Is there functional redundancy? And f) how are LC3/GABARAP family proteins selectively recruited to the required site? In this manuscript, we address these questions by assembling the current knowledge on processing, regulation, function and specificity of the LC3/GABARAP family proteins. By connecting the data described in literature, we highlight the underlying complexity in the mammalian autophagy machinery to permit a more complete interpretation of new experimental results in the future.

Together, the provided data indicates that not all LC3/GABARAP family proteins are redundant in the autophagy process and each has a unique (autophagy independent) important function for cell homeostasis.

Functions of LC3/GABARAP family proteins in autophagy (table 1)

1. Initiation, elongation and closure of the autophagosome

Whether the membrane for nucleation is formed *de novo* or is derived from an organelle is not fully understood yet. Membrane components can be derived from endoplasmic reticulum (ER), Golgi complex, endosomes, plasma membrane and ER-mitochondria contact sites and may be driven by the distance of the source to the initiating membrane (reviewed in (11)). Recently, it has become clear that the Unc51-like kinase 1 (ULK1) functions as a facilitator of phagophore formation. ULK1 is present in complexes consisting of ULK1, ATG13, FIP200 and ATG101 and can alter the complex's activity through increased kinase activation (12) and is shown to be crucial during stress conditions (13). ULK1 phosphorylates beclin 1 (BECN1), a member of a second crucial autophagy-related complex and implicated in ER-derived phagophore formation (14). Part of this complex is a phosphoinositol 3 kinase (PI3K; Vps34) which phosphorylates phosphospatidylinositol (PI) at position 3 to produce PI(3)P. PI(3)P serves as a docking site for effectors to further mediate phagophore maturation and elongation (15). Pull-

down of ectopically expressed LC3/GABARAP family proteins revealed that GABARAP and GABARAPL1 display highest affinity to ULK1 and LC3B the lowest. This suggests that GABARAP subfamily proteins can, more efficiently than LC3B, serve as scaffolding proteins by recruiting ULK1 and beclin 1 (complex) to the site of nucleation (16) (Fig 1A). Whether this indicates that phagophore development or cargo engulfment is dependent on GABARAP proteins or that GABARAP proteins allow selective cargo engulfment remains unknown.

After elongation autophagosome closure the autophagosome is formed (Fig. 1C and D) and requires fusion events of both the inner and outer membrane leaflets (Fig. 1D). In eukaryotic cells, specialized functions seem to be attributed to specific LC3/GABARAP family proteins. For example, cells deficient in the LC3 subfamily (LC3A, LC3B, LC3C) or GABARAP subfamily (GABARAP, GABARAPL1, GABARAPL2) display impaired autophagosome formation (17). Yet, knockdown of the LC3- or GABARAP subfamily proteins leads to different autophagy impairment phenotypes, as deficiency in the LC3 subfamily (LC3A, LC3B and LC3C simultaneously) leads to generation of smaller autophagosomes whereas deficiency of GABARAP subfamily (GABARAP, GABARAPL1 and GABARAPL2 simultaneously) leads to biogenesis of larger autophagosomes. Re-expression of LC3B or GABARAPL2 is sufficient to rescue autophagy. Here, LC3B rescues phagophore elongation and GABARAPL2 mediates closure of the vesicle(17). These results are in line with observations in cell free systems where the amino-terminal α -helices of GABARAPL2 and LC3B promote tethering and fusion of membranes. Although the fusion activity of GABARAPL2 and LC3B are similar, the fusion activity of LC3B is mediated by ionic interactions, whereas those of GABARAPL2 are mediated by hydrophobic interactions(18). Interestingly, GABARAP family proteins are present in an open and closed conformation. Herein, the open form has a protruding amino-terminal helix with hydrophobic residues that enables penetration into the adjacent membrane and interacts with the hydrophobic center of the lipid bilayer (19). In contrast, LC3B interacts with the lipid head group (18). Together, these events can bring together (extending) membranes and lead to phagophore elongation (hemifusion, Fig. 1C) and closure (fusion, Fig. 1D). Whether the transition from elongation to closure is merely chance or is an orchestrated event when the cargo is engulfed remains unclear. These data suggest little redundancy between LC3/GABARAP family protein function as functions appear to be inherent to their structural characteristics.

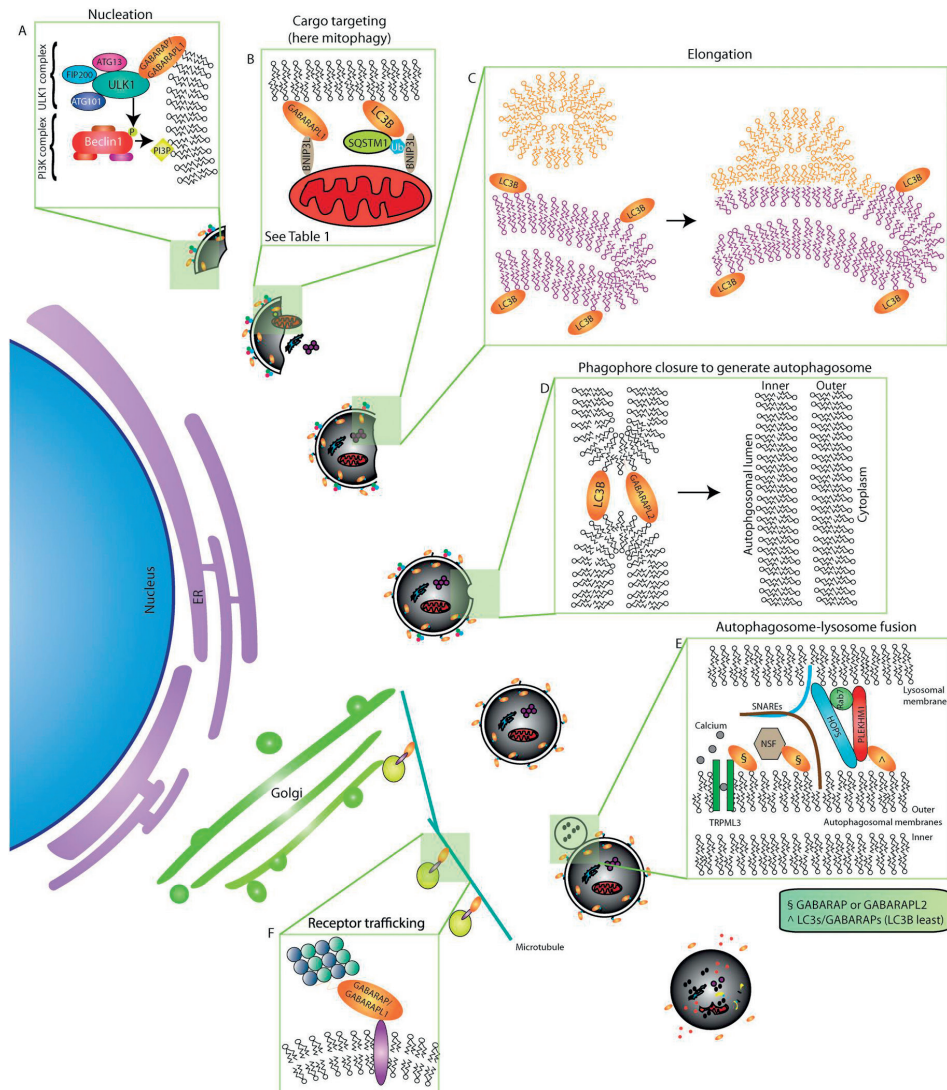


Figure 1. Involvement of LC3/GABARAP family proteins in (non-)autophagy related processes.

Autophagy initiation involves the formation of a phagophore. This nucleation phase **(A)** is commenced by two protein complexes, ULK1 complex and Beclin1 complex. Herein, the first can be localized to the phagophore by binding predominantly GABARAP/GABARAPL1. The ULK1 complex activation status influences the rate of autophagy by Beclin1 phosphorylation. The phagophore can engulf content either selectively or non-selectively. Selectivity in cargo tethering to the phagophore **(B)** is established by the LC3/GABARAP family members that have different intrinsic properties that allows the binding linker proteins. Here mitophagy is illustrated by the protein BNIP3L/Nix at the mitochondrial membrane that can bind GABARAPL1 directly or be ubiquitinated by SQSTM1 mediated recognition. Elongation **(C)** requires the delivery of membrane components (orange) to the growing phagophore (purple). LC3B is the main family protein to effectuate elongation. Phagophore closure **(D)** generates a closed double membraned vesicle. Fusion of both membrane leaflets is

required for closure that is thought to be done by LC3B and GABARAPL2 in a cooperative fashion. Subsequent fusion of the autophagosome with a lysosome (**E**) is required to acquire the degradative enzymes and acidification of the autophagosomal lumen for cargo degradation. This fusion event requires GABARAP and GABARAPL2 by their interaction with NSF and the calcium channel TRPML3, which both facilitate fusion. Moreover, all LC3/GABARAP proteins (notably LC3B the least) interact with PLEKHM1 that mediates bridging both vesicles. Besides autophagy, recent findings indicate the relevance of, in particular, GABARAP family proteins in receptor trafficking (**F**). These are involved as adaptor proteins connecting the receptors in golgi-derived vesicles to tubulin fibers (also see table 1).

II. Cargo targeting

Initially, autophagy was considered a process of non-selective degradation. However, increasing evidence indicates that cargo can selectively be targeted for degradation. Selective autophagy has been described for aggregated proteins (aggrephagy), mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy), lipid droplets (lipophagy) and pathogens (xenophagy); however much remains to be elucidated regarding the regulation and molecular mediators involved. For the engulfment of several specific substrates, LC3/GABARAP family proteins play a pivotal role through interaction with autophagy adaptor proteins such as sequestosome 1 (SQSTM1/p62, hereafter referred to as SQSTM1), neighbor of BRCA1 gene 1 (NBR1), calcium binding and coiled-coil domain 2 (NDP52/ CALCOCO2), BCL2/adenovirus E1B 19kDa interacting protein 3-like (NIX/BNIP3L), optineurin (OPTN), and WD repeat and FYVE domain containing (ALFY) (table 1). These are able to bind both the ubiquitinated substrate/cargo and LC3/GABARAP family proteins through their LC3 interacting regions (LIRs)(20) and facilitate cargo-tethering to the luminal side of the phagophore. The combination of LC3/GABARAP protein and adaptor protein is therefore essential in cargo selection, targeting and degradation.

Aggrephagy: Protein aggregates and peroxisomes are degraded through autophagy. In this SQSTM1 plays an essential role (21). Although SQSTM1 displays no differences in LC3/GABARAP family protein affinity, only lipidated LC3B is able to recruit SQSTM1 to autophagosomes(22). Interestingly, ALFY (an autophagy receptor required for aggrephagy(23)), co-immunoprecipitates predominantly with GABARAP. The binding of ALFY to GABARAP is indispensable for the recruitment of LC3B-positive structures and the degradation of SQSTM1 structures (24). This suggests that GABARAP and LC3B function in a cooperative fashion during aggrephagy to establish a selective form of autophagy. Recently, a new class of ubiquitin-atg8 receptors (CUET proteins) have been identified. The yeast CUE-ubiquitin-binding-domain protein Cue5 and its human homolog toll interacting protein (TOLLIP) mediate the clearance of polyQ protein aggregates. PolyQ proteins contain a polyQ tract, as a result of trinucleotide repeat, cytosine–adenine–guanine (CAG) expansions, which encodes glutamine. Their aggregates are linked to neurodegenerative disorders like Huntington’s disease. TOLLIP contains two LIR motifs for interaction with LC3B The interaction with LC3 allows spatial organization of TOLLIP

recruitment and subsequent ubiquitination through CUE recruitment and degradation of the aggregate. Interactions with other members of the LC3/GABARAP proteins family remain matter of investigation (25).

Xenophagy: In the elimination of intracellular pathogens, LC3C is uniquely required due to its strong preferential binding to the autophagy receptor, NDP52/CALCOCO2. *Salmonella* Typhimurium, damages its surrounding vacuole membrane after invasion, leading to exposure of host glycans and microbial carbohydrates. The ‘vesicle damaging receptor’ galectin 8 /LGALS8 recruits NDP52 and results in activation of bacterial autophagy(26). The binding of LC3C to NDP52 leads to the engulfment of the bacterium in the autophagosome. After entrapment, NDP52 is required for the maturation and degradation of *Salmonella* Typhimurium-containing autophagosomes through interaction with, LC3A, LC3B and GABARAPL2 (27,28). The binding of LC3C and the other LC3/GABARAP family proteins is dependent on the presence of two distinct LIRs. Because of the essential role of “LC3C-LIR”, its role in xenophagy is non-redundant. In line, Galectin8, LC3C or NDP52 knockdown cells are defective in blocking proliferation of *Salmonella* (29), indicating a unique cooperation in xenophagy. Additionally, upon phosphorylation by TANK binding kinase 1 (TBK1) to enhance LC3 binding affinity, the autophagy receptor optineurin (OPTN) targets similar cytosolic bacteria as NDP52. OPTN mutants that are deficient in LC3 binding or silenced OPTN results in impaired degradation of ubiquitylated *Salmonella enterica* (30). In addition to *Salmonella*, P62 and NDP52 also sequester intracytosolic *Shigella* and the membrane remnants to the autophagosome for degradation (31,32)

Tax1 binding protein1 (TAX1BP1) is also involved in clearance of intracellular *Salmonella* and also depends on ubiquitination of the substrate. TAX1BP1 not only binds to the LC3 subfamily members LC3B and LC3C but also interacts with GABARAPL1 and GABARAPL2. In contrast to NDP52 and OPTN, there is no preference in binding affinity for LC3B of LC3C although it shares the same canonical LIR motif as OPTN and NDP52 (33).

Furthermore, the TRIM family plays a role in antiviral defense by targeting viral capsid proteins. Mandell et al found that the TRIM family, and especially tripartite motif containing5 (TRIM5 α), interacts preferably with GABARAP and GABARAPL1, and to a lesser extend LC3A, LC3C and GABARAPL2. Although GST-pulldown assays show only a weak interaction with LC3B, co-immunoprecipitation studies and immunostainings show that LC3B may nonetheless be in complexes with TRIM5 α . TRIM5 α contains two LIR motifs. Mutation of these motifs results prevented the HIV capsid protein p24 from degradation (34).

Glycophagy: Several myopathies are associated with defects in autophagic and lysosomal degradation of glycogen, illustrating its importance in maintaining glycogen homeostasis (35). In normal functioning, glycogen is hydrolyzed to glucose by lysosomal α -glycosidase and is therefore an important source of energy. Starch-binding domain containing protein 1 (STBD1/genethonin 1) binds glycogen and anchors it to mem-

branes, thereby affecting its cellular localization and its intracellular trafficking to lysosomes (36). STBD1 interacts with GABARAPL1 and to lesser extent GABARAP (37). Mutations and deletions in STBD1's motif to bind GABARAP subfamily proteins indicate that this motif is required for this GABARAPL1-mediated engulfment of glycogen into vesicles (38). Although the interaction has been well established, the exact role of GABARAPL1 in glycophagy remains to be elucidated in functional assays.

Mitophagy: Mitochondrial metabolism leads to the formation of by-products that lead to oxidative stress, DNA damage and mutations and increases in aged and dysfunctional mitochondria. Sustaining a healthy population of mitochondria is therefore critical to maintain cellular homeostasis and prevent accumulation of damaging agents. The turnover of mitochondria is autophagy-dependent and inhibition leads to accumulation of mitochondria and excessive exposure to reactive oxygen species (ROS) (39). Like other substrates, mitochondria can be ubiquitinated for degradation. Interestingly, there is some overlap in receptors involved in the recognition of other ubiquitinated substrates e.g. in xenophagy. After depolarization of the mitochondrial membrane, PINK accumulates in the outer mitochondrial membrane and recruits NDP52 and OPTN, but not p62, to activate mitophagy independently of PARKIN. Interestingly, in knockout models of the 5 mitophagy receptors (TAX1BP1, optineurin, NBR1, p62 and NDP52), Lazarou et al found that LC3C is recruited by TAXBP1 during mitophagy and LC3B by OPTN and NDP52. Furthermore they observed that GABARAP family proteins are not recruited to mitochondria, suggesting that they are not actively involved in mitophagy (40).

In contrast, Novak et al found that GABARAPL1 colocalizes with depolarized mitochondria during basal, starvation and apoptotic conditions (41-43). GABARAPL1 deficiency attenuates autophagic flux and accumulates SQSTM1 including mitophagy and results in accumulation of damaged mitochondria (43). Whether GABARAPL1 is directly involved in mitophagy or influences mitophagy through modulation general autophagy activity remains matter of investigation. Nevertheless, the direct colocalization of GABARAPL1 with mitochondria and its interaction with another important factor in mitophagy, BNIP3L/Nix suggests a direct role for GABARAPL1 in mitophagy (41) (Fig. 1B).

FUNDC1, an integral mitochondrial outer-membrane protein, acts as a specific receptor for mitophagy in an ubiquitin independent manner. In response to hypoxia FUNDC1 is able to bind LC3 proteins -A and -B and GABARAP and GABARAPL2 via its typical LIR domain ((YxxL). Upon hypoxia stimulation, Scr kinase is inactivated and the FUNDC1 LIR domain is dephosphorylated, resulting in an increased colocalization and interaction FUNDC1 and LC3B (44). Nix and its homolog Bnip3 also play a role in hypoxia induced mitophagy. Bnip3 and Nix, both members of the BCL2 family, are anchored in the outer mitochondrial membrane via their C terminal transmembrane domains, BNip3 and Nix contain an identical LIR motif (WxxL). Phosphorylation of the ser17 and ser24 specifically promotes LC3B and GABARAPL2 binding to Bnip3(45).

Ubiquitin-dependent cargo recognition can be executed by multiple different receptors. Intriguingly, there seems to be an underlying layer of complexity when it comes to LC3/GABARAP proteins family member recruitment. For instance, during the clearance of cytosolic salmonella, TAX1BP1 recruits members of the LC3 and GABARAP protein subfamily. However, during mitophagy, TAX1BP1 selectively recruits LC3C and no GABARAP proteins. It remains matter of investigation of how selective autophagy receptors recruit specific LC3/GABARAP proteins family members to targeted cargos in an ubiquitin (in-) dependent manner.

III. Autophagosome-lysosome fusion

The autophagosome can fuse with a lysosome directly to form the autolysosome or fuse with multiple components of the endocytic pathway including early and late endosomes or multivesicular bodies (MVBs) to generate amphisomes(46). Fusion of autophagosomes or amphisomes with acidic lysosomes is required for content degradation. RAB7, a member of a family of small GTPases, is important in lysosome fusion (47) through interaction with homotypic fusion and protein sorting (HOPS) complex. HOPS functions as a guanine nucleotide exchange factor (GEF) to facilitate RAB7 activation and is a downstream effector of RAB7 to promote membrane tethering. For this, HOPS interacts with SNAP (soluble NSF (N-ethylmaleimide-sensitive factor)) attachment protein) receptors (SNAREs) that can interact with other SNAREs through coiled-coil type interactions to facilitate membrane fusion. Full fusion of the autophagosome with the vacuole requires NSF/SEC18 (48), which changes the SNARE's conformation and allows binding to its interactor (49). Interestingly, GABARAPL2 and GABARAP are both able to interact with NSF (50,51). In addition, GABARAPL2 interacts with transient receptor potential cation channel, mucolipin subfamily, member 3 (TRPML3) (52). This is a Ca^{2+} permeable cation channel that is proposed to facilitate fusion events by promoting interbilayer contact (53). Thus, GABARAPL2 (and GABARAP) facilitate fusion by scaffolding NSF and TRPML3 at the location of fusion (Fig. 1E).

In addition, pleckstrin homology domain-containing family M member 1 (PLEKHM1), a RAB7 effector which also binds components of the HOPS complex can function as a bridge between autophagosomes and lysosomal membranes. Pulldown analysis demonstrated that PLEKHM1 interacts with all LC3/GABARAP family proteins. Notably, the yield of LC3B was lowest (54) suggesting that autophagosome-lysosome fusion is more dependent on other LC3/GABARAP family proteins.

In this section the different involvement and functions of the LC3/GABARAP family proteins in phagophore formation, elongation and closure, autophagosome-lysosome fusion and selective forms of autophagy have been highlighted. Although structurally these family proteins share high homology, each one seems to fulfill (a) unique function(s) in the execution of autophagy.

Table 1: Summary of specialized functions of LC3/GABARAP family proteins in autophagy

Subfamily	Gene	Chromosome location	Gene size (kb)	Protein	Iso forms	Component	Mechanism	Ref
LC3	MAP1LC3A	20q11.22	1.0	LC3A-a*	2	Anterograde trafficking	Interaction with the RAB7 effector FYCO1 and PLEKHM1	(131, 132)
				LC3A-b*		Fusion Xenophagy Mitophagy	Interaction with TRIM5α for viral clearance Recruitment by FUNDC1	(34) (44)
	MAP1LC3B	16q24.2	10.7	LC3B	1	Elongation	Covalent attachment of PE by the ubiquitin-like conjugation system.	(15,16)
						Recruitment	Recruitment of p62/SQSTM1 positive structures to the autophagosome.	(22)
GABARAP	MAP1LC3B2	12q24.22	0.8	LC3B2	1	Fusion	Phosphorylation by STK3/STK4	(127)
						Anterograde trafficking	Interaction with the RAB7 effector FYCO1 and PLEKHM1	(131) (54)
						Aggrephagy Xenophagy Mitophagy	Interaction with TOLLIP Interacts with cargo receptor TAX1BP1	(25) (33)
							Recruitment by optineurin, NDP52 and FUNDC1	(40,44)
	MAP1LC3C	1q43	2.8	LC3C	1		So far, no functional role in autophagy reported.	(73)
						Xenophagy	Binding ubiquitinated bacteria receptor NDP52	(26,29)
						Fusion	Interaction with PLEKHM1	(54)
						Xenophagy	Interacts with cargo receptor TAX1BP1	(33)
	GABARAP	17p13.1	1.5	GABARAP	1	Xenophagy	Interaction with TRIM5α for viral clearance	(34)
						Mitophagy	Recruitment by TAX1BP1	(40)
						Initiation Fusion	Interacts with ULK1, an important mediator of nucleation	(16) 38,39
							Interacts with NSF and PLEKHM1	(50) (54)
						Anterograde trafficking	Phosphorylation by STK3/STK4 Interaction with the RAB7 effector FYCO1	(127)
							Potentially reprogram endocytic trafficking events to facilitate autophagy	(131)
						Aggrephagy Xenophagy	Interaction with ALFY and NBR1	(24,133)
						Mitophagy	Interacts with cargo receptor TAX1BP1 Interaction with TRIM5α for viral clearance	(33) (34)

GABARAPL1	12p13.31	8.7	GABARAPL1 GEC1	1	RAC1 signaling Initiation Fusion Mitophagy Glycophagy Xenophagy	Recruitment by FUNDC1	(44)
						Interaction with TIAM1	(134)
						Interaction with ULK1	(16)
						Potentially reprogram endocytic trafficking events to facilitate autophagy	
						Interaction with PLEKHM1	(41)
						Interaction with BNIP3L/Nix	(38)
						Interaction with STBD1	(34)
						Interaction with TRIM5α for viral clearance	
						conjugation to PE	(15,16)
						Interaction /scaffolding NSF and TRPML3	(50,52)
GABARAPL2	16q22.1	10.9	GABARAPL2 GATE-16 GEF2	1	Vesicle closure Fusion Trafficking Xenophagy Mitophagy	Phosphorylation by STK3/STK4 and PLEKHM1	(54,127)
						Potentially reprogram endocytic trafficking events to facilitate autophagy	
						Interacts with cargo receptor TAX1BP1	(33)
						Interaction with TRIM5α for viral clearance	(34)
						Recruitment by FUNDC1	(44)

* splice variants

Functions of LC3/GABARAP family proteins independent of autophagy?

Although all LC3/GABARAP family proteins seem to fulfill (specific) roles within the execution of autophagy, some of the family proteins also fulfill functions that appear canonical autophagy-independent. Several LC3/GABARAP family proteins have been implicated in receptor-trafficking, while others have been implicated in tumour-suppressive functions that appear autophagy independent.

1. Intracellular trafficking

Initially, the GABARAP protein family was described in intracellular transport of the GABA (A) receptor (GABAAR)(55). Further studies revealed that GABARAP also mediates membrane expression of the angiotensin II receptors (AGTR) (56,57), the human kappa opioid receptor (hKOPR) (58) and the transient receptor potential cation channel sub-family V member 1 (TRPV1) (59).

After assembly in the ER, GABAARs are transported to the golgi and are incorporated into transport vesicles (55). To facilitate receptor transport from the golgi to the plasma membrane, GABARAP interacts directly with the $\gamma 2$ subunit of the GABAAR. Here, GABARAP possibly serves as a cargo-receptor required for GABAAR incorporation into the transport vesicle. Interestingly, GABARAP knockout mice show no defect in GABAAR trafficking, indicating that other molecules, such as GABARAPL1 can compensate for the loss (60).

Transport vesicles migrate along microtubules towards their destination. Antero-grade transport, trafficking towards the plasma membrane, is facilitated by motor proteins of the kinesin family. Kinesin family member 5A (KIF5A) and kinesin family member 21B (KIF21B) control GABAAR surface expression via interaction with GABARAP (61,62). Yeast two hybrid screens also identified interactions with GABARAPL1 and GABARAPL2, however if these interactions are functional, remain elusive (61). In addition, Monastyrska et al., proposed that vesicles exposing LC3/GABARAP protein family members on their surface, can be 'docked' on the microtubule by their highly positively charged N-terminal region tubulin binding motif until further processing (63). Interestingly, this tubulin binding motif is conserved throughout the whole LC3/GABARAP protein family (64). Furthermore, the interaction of GABARAP with the endocytosis associated protein clathrin suggests that GABARAP may also be involved in the receptor internalization (65). Hence, Lainez et al. showed that internalization and recycling of the TRPV1 is promoted by GABARAP, without altering the degradation rate of the receptor (59).

When transport vesicles reach their destination, membrane fusion is required for further processing. Fusion of transport vesicles with target membranes is mediated by SNARE proteins. *N*-ethylmaleimide-sensitive factor (NSF) plays a role in the dissociation of SNARE complexes after membrane fusion, allowing them to be recycled for subsequent rounds of fusion. NSF also plays a role in the sorting of receptors into transport

vesicles and can bind GABARAP and the GABAAR (66). Interestingly, GABARAP is not found at GABAergic sites, but mainly in intracellular vesicles and at the end of golgi stacks where transport vesicles bud and fuse, suggesting its main role is transportation of these vesicles and not anchoring on the plasma membrane (66,67). Although, increasing GABARAP expression leads to enhanced GABAAR expression at the plasma membrane (68), Gephyrin is required for GABAAR clustering (69). Since GABARAP is the only molecule that is able to bind both tubulin, gephyrin and the GABAAR, GABARAP may be the adaptor molecule between gephyrin and the GABAAR (70). Like all members of the LC3/GABARAP family, the C-terminal glycine of GABARAP can be conjugated to PE to establish its incorporation in the membrane. This modification is required for transportation of the GABAAR (58), but is not required for trafficking of the Angiotensin II type A1 receptor (57).

In addition to GABARAP, GABARAPL1 is required for anterograde transport of the kappa opioid receptor (KOR). Knockdown of either GABARAPL1 or GABARAP decreases the surface expression of kappa opioid receptor (58). Recently, we showed that the expression of GABARAPL1 during hypoxia is not restricted to the cytoplasm, but also localizes at the intraluminal side of the plasma membrane of cancer cells. At these sites, GABARAPL1 co-localizes with epidermal growth factor receptor (EGFR). GABARAPL1 knockdown cells display reduced EGFR expression at the plasma membrane, whereas total EGFR levels are not altered (71). Similar results were also observed for carbonic anhydrase IX (CAIX), a hypoxia inducible factor-1 (HIF-1) transcriptional target (71), suggesting that GABARAPL1 plays a role in membrane expression of proteins during hypoxia.

In contrast, GABARAPL2 is a membrane transport enhancer involved in intra-Golgi trafficking (72) (Fig. 1F). The Golgi complex is considered the central component of the secretory pathway required for lipid and protein modification, classification, packaging into specific carriers and delivery. GABARAPL2 stimulates the ATPase activity of NSF and is capable of interacting with GOS-28, a Golgi specific SNARE. The coupling between NSF activity and SNAREs activation allows GABARAPL2 to modulate intra-Golgi transport (51).

As summarized above, the GABARAP family proteins facilitate receptor trafficking, actions that appear independent of autophagy. This illustrates the complexity of data interpretation in (genetic) models that change expression of these factors.

II. Oncogene/Tumour suppressor

LC3A is expressed in two isoforms, LC3A-a and -b (NP_115903 and NP_852610). They differ in amino-terminus sequence as a result of a different transcriptional start site. LC3A-a, but not LC3A-b, is turned over by lysosomes and whereas LC3A-a is ubiquitously expressed in most tissues, LC3A-b expression is low in general. Interestingly, LC3A-a expression, but not LC3B, is silenced in 45.5% of human cancer cell lines originating

from various tissues. Methylation of the CpG island proximal to the LC3A-a transcriptional start site is observed in human cancer cell lines tested by COBRA (combined bisulfite restriction analysis), while no aberrant methylation was observed in LC3A-a expressing lines. Interestingly, LC3A-a re-expression in LC3A-a silenced cells decreases xenograft growth, suggesting a role for LC3A-a in tumour progression (73).

VHL (Von hippel Lindau) syndrome is an autosomal dominant disease cancer syndrome associated with loss of E3 ligase/tumour suppressor gene VHL which increases predisposition to many tumours including renal cancer. In the majority of spontaneous clear cell renal cell carcinomas (ccRCC) VHL is deleted (74). In renal tumours without loss of VHL, high levels of LC3C are observed. Knockdown of LC3C in these VHL expressing ccRCC cells increases the incidence of orthotopic tumour formation. Interestingly, in these models tumour progression is associated with increased LC3B-mediated autophagy. While LC3B-mediated autophagy is required for tumour progression, LC3C acts as a tumour suppressor (75). This suggests that LC3C functions here independent of canonical (autophagosomal-lysosomal degradation) and LC3B-mediated autophagy to maintain homeostasis. Interestingly in colorectal cancer high LC3B expression is correlated with improved survival after surgery (76). This suggests that LC3B associated autophagy can promote or repress tumorigenic events dependent on the tumour site and potential other mechanisms at play.

Recently, GABARAP has been indicated as a tumour suppressor gene. Compared to normal breast tissue, primary breast cancer tissues contain lower GABARAP mRNA and protein levels, this is in agreement with results observed in cell lines. Ectopic expression of GABARAP gene in low-expressing breast cancer cell lines reduced tumour growth rate (77). Moreover, tumour incidence induced by mutagenic and immunotoxic 7,12-dimethylbenz(a)anthracene (DMBA) treatment was reduced in GABARAP knock out (KO) mice. Interestingly, injection of GABARAP proficient melanoma cells in GABARAP knock out mice led to reduced tumour growth, supporting the tumour suppressive role of GABARAP (78). This tumour suppressive feature of GABARAP could be mediated through direct effects on autophagy and/or in an indirect fashion by controlling receptor trafficking such as EGFR (79) (71)).

III. ER –associated degradation (ERAD)

The ER requires a quality control system to ensure correct protein folding. Misfolded proteins are selected for degradation by processes collectively referred to as ER-associated degradation (ERAD). At steady state, the effector proteins in ERAD (including EDEM1 and OS9) are removed rapidly from the ER to establish low ER residence, defined as ERAD tuning. EDEM1 turnover is reduced in mouse embryonic fibroblasts lacking ATG5 or treated with chloroquine. Gradient separated fractions showed that a small fraction of EDEM1 is associated with bulk ER; however, the majority of EDEM1 is present in other fractions, fractions with LC3-I (80). This suggests that LC3 coated vesicles

that differ from autophagosomes (as LC3-I rather than LC3-II is associated) are required for the disposal of EDEM1 in the process of ERAD tuning.

IV. LC3-associated phagocytosis

During phagocytosis a cell engulfs particles (as cells or pathogens) to create an intracellular vesicle known as a phagosome. Recently it was observed that these single membrane vesicles can express LC3(81). The process is referred to as LC3-associated phagocytosis, or LAP. LC3 lipidation is required for its association with these phagosomes, and is dependent on an array of autophagy related proteins including ATG5, ATG7, Beclin1, ATG12, and ATG16L (81-83). However, other autophagy related proteins seem to be dispensable for LAP including ULK1/2, ATG13, FIP200 and ATG14(83). In addition, LAP specific proteins (dispensable for autophagy) are Rubicon (a Beclin1 interacting protein) and ROS-producing NADPH oxidase 2 (NOX2) (84). Recently, Martinez et al exploited this segregation in required proteins between autophagy and LAP by inducing LAP specific abrogation in immune cells and challenging mice with apoptotic cell injections. The phenotype that these mice presented included accumulation of lymphocytes, monocytes, activated CD8+ cells, developed autoantibodies and kidney damage with increased interferon gamma expression. This phenotype resembles aspects of systemic lupus erythematosus (SLE) patients and is the result of increased pro-inflammatory cytokine secretion. Therefore, LAP was suggested to be required to protect against certain autoimmune diseases (85). Nevertheless, phagosome maturation can occur independent of LC3(86), thus requirement of LC3 and other ATG proteins for phagosome maturation is possibly condition dependent. Nevertheless, LAP facilitates antigen presentation on major histocompatibility complex (MHC) class II molecules (87).

V. motility and growth

Ras-related C3 botulinum toxin substrate 1 (RAC1) mediates cell motility and growth. RAC1 activity is controlled by the guanine exchange factor TIAM1. The recently discovered CUL3 KBTBD6/KBTBD7 ubiquitin ligase complex controls TIAM1 abundance by ubiquitination and targeting for proteasomal degradation. Interestingly, ubiquitination of TIAM1 depends on binding to GABARAP, located on GABARAP positive vesicles, resulting in restriction of RAC1 signaling to specific sites in the cell. For binding to GABARAP, KBTBD6/7 contains a LIR motif to bind preferably to GABARAP proteins rather than the LC3 subfamily. Furthermore, the authors show that these GABARAP positive vesicles are not associated with autophagosome formation or degradation, suggesting a vesicle population distinct from autophagosomes (88).

VI. Maintenance of ERES sites

Endoplasmatic reticulum exit sites (ERES) are highly specialized sites that allow the export of proteins into COPII vesicles. In addition to their role in protein export, ERES are involved in the biogenesis of autophagosomes possibly through the delivery of membrane- and other components that are essential in the formation of autophago-

somes or act as scaffold sites where the autophagosomes may be formed (reviewed in (89)). COPII consists of several protein including the cargo adapters SEC23 and SEC24 (90). In conjunction with lipidated LC3C, Tectonin- β -propellor containing protein 2 (TECPR2) associates with SEC24D and allows for efficient ER cargo export. Hence, either knockdown of TECPR2 or LC3C results in delayed delivery of cargo from the ER to the golgi (91). In addition, the maintenance of ERES via TECPR2 and LC3C is required to autophagosomes formation, failure to maintain ERES sites results in impaired autophagosomes development (91).

Given the high complexity and involvement of the LC3/GABARAP family proteins in these diverse cellular processes, it is likely that these family proteins may be involved in other momentarily unidentified processes. Nevertheless, substantial evidence has shown that the family proteins are also involved in autophagy unrelated processes. Experiments where these family proteins are knocked down should therefore interpreted with caution.

How the LC3/GABARAP family proteins may selectively be recruited

The LC3/GABARAP protein family fulfills a variety of functions in intracellular trafficking and autophagy. As discussed above, most of these functions appear family protein specific. Interestingly, the functionality and activation of the LC3/GABARAP family proteins at first glance appears to be mediated by comparable enzymatic reactions (terminal glycine exposure, PE-conjugation). To achieve differences and effective recruitment of the required LC3/GABARAP family protein, the cell exploits affinity and specificity differences for ATG proteins which allow complex selection and recruitment of specific LC3/GABARAP family proteins. In this section, the complex recruitment and availability that is required for autophagy execution is discussed.

1. LC3/GABARAP family protein activation

LC3/GABARAP family proteins are present in cytoplasmic and membrane-bound isoforms; indicated as -I and -II (for LC3B; LC3B-I and -II) respectively. In yeast, covalent conjugation of a PE-group to Atg8 is required for membrane incorporation. In the Atg8 conjugation reaction, Atg4, Atg7 and Atg3 are crucial(9). For the mammalian orthologues, similar mechanisms have been identified for LC3B, GABARAP, GABARAPL1 and GABARAPL2 (92,93). Whether LC3A and LC3C require ATG4 processing and ATG7 and ATG3 interactions for PE conjugation has not yet been demonstrated.

A series of modifications is required for the efficient conjugation of PE and the correct localization of the LC3/GABARAP family proteins. Members of the ATG4 protease family cleave the pro-form to expose the conserved glycine (isoform-I). Subsequently, the E1 activating enzyme ATG7 catalyzes acyl adenylation of the carboxyl-terminus and

is then transferred to the E2 conjugating enzyme, ATG3 and, in turn, mediates the covalent conjugation to PE (isoform-II)(94). Although each of the LC3/GABARAP family proteins undergoes this type of conjugation, differences in efficiency and specificity exist. For example, conjugation is most efficient for GABARAPL1, followed by GABARAPL2 and least efficient in LC3B (95). This interpretation is based on the ratio I/II in cell-free assays exposing isoform-I to ATP, ATG7, ATG3 and liposomes that are devoid of additional regulatory mechanisms or post-translational modifications. Nevertheless, this setup provides assessment of the systems potential for individual LC3/GABARAP family protein conjugation. These differences indicate that the ratio in LC3/GABARAP family proteins presentation to the ATG7-ATG3 process has a major impact on the generation of membrane associated (-II) forms.

In addition to LC3/GABARAP family protein conjugation, autophagy relies on another conjugation reaction to create the ATG12-ATG5-ATG16(L) complex. For their interaction, ATG12 is activated by the E1 activating enzyme ATG7. Hereafter, ATG12 is transferred to ATG10 (E2 conjugating enzyme) and conjugated to ATG5. ATG5 interacts with ATG16(L) to form the complex, which can form a tetramer *via* ATG16 homodimerization (reviewed in (96)). The complex fulfils multiple functions. First, it is involved in determination of the site for LC3/GABARAP family protein conjugation to PE by ATG3. In yeast, the Atg12-Atg5 conjugate promotes the transfer of Atg8 from Atg3 to the substrate, PE, by stimulating the activity of Atg3 (8). Also in mammalian cells, localization ATG12-ATG5-ATG16L determines the site of LC3B conjugation (97). The direct interaction of ATG12 and ATG3 (98) further indicates that ATG12 recruits ATG3 to determine localization of LC3/GABARAP family protein membrane incorporation. Nevertheless, the ATG12-ATG5 conjugate, but not ATG12 alone, is able to facilitate this LC3/GABARAP family protein PE-conjugation (98). Second, the ATG12-ATG5 conjugate interacts with TECPR1 (a lysosomal resident protein) which is crucial for autophagosome-lysosome fusion (99). Third, the yeast Atg12-Atg5-Atg16 complex and Atg8 cooperate into a self-organizing system to form a protein mesh (scaffold) causing stabilization of the membrane (100). This interaction is mediated through a non-canonical Atg8 binding motif in Atg12, antiparallel arrangement of two Atg16 dimers and the ability of Atg5 to bind membranes and interact with Atg16(L) (100). Interestingly, in mammalian cells GABARAPL1 is observed at the plasma membrane (101) and could indicate a role for GABARAPL1 in membrane stabilization. It is however still unclear whether the ATG12-ATG5-ATG16 complex is capable of interacting with other LC3/GABARAP family proteins than LC3B.

The LC3/GABARAP family proteins fulfil unique functions, but appear to be regulated by similar mechanisms. Interestingly, the machinery displays differential conjugation efficiency that can be of importance to orchestrate the functional complexity among the LC3/GABARAP family proteins.

1.i. ATG4 cysteine protease family – four isoforms varying in homologue selectivity

Directly after production, pro-LC3B is cleaved at glycine 120 generating isoform I. This priming is comparable for all LC3/GABARAP family proteins and is ATG4 dependent. ATG4 is a class of cysteine proteases with four isoforms in humans; ATG4A, -B, -C and -D. Most information is available on ATG4B which is capable of cleaving LC3B (42,102-104), GABARAP (102-104), GABARAPL1 (42,104) and GABARAPL2 (42,102,103). ATG4A displays different substrate specificity and cleaves GABARAPL2 most efficient, followed by GABARAP, and LC3B the least (102,104). Interestingly, overexpression of dominant negative ATG4A mutant (ATG4A^{C77A}) increases the number of unclosed autophagic membranes (17) and is in line with the role for GABARAPL2 in vesicle closure (17,18). Interestingly, ATG4C and ATG4D contain a caspase 3 cleavage domain (DEVD) and, compared to full-length ATG4D, its cleaved form has higher priming and delipidating activity, predominantly directed to GABARAPL1. ATG4D silencing sensitizes HeLa cells to starvation-induced cell death, suggesting that ATG4D-dependent autophagy contributes to cellular survival. Interestingly, cleaved ATG4D is recruited to mitochondria (42) and may mediate recruitment of GABARAPL1 for (dysfunctional) mitochondria clearance (43). Together, this implicates that specific LC3/GABARAP family protein activation and recruitment can be regulated at the post-translational level through activation or expression of ATG4 isoforms.

1.ii. ATG3 and ATG12-ATG5-ATG16(L) dynamics in LC3/GABARAP family protein recruitment

As LC3/GABARAP family proteins display different functions, recruitment and activation at the site of requirement is therefore important. Directing specific family proteins to the 'flat' membrane or the highly curved extending tips of the phagophore is crucial for elongation and closure. In addition, as LC3/GABARAP family proteins are required for specific types of autophagy, selective recruitment is essential. For example, autophagosomes directed for lysosomal degradation or receptor trafficking from Golgi to the plasma membrane (GABARAP and GABARAPL1-mediated anterograde transport (57,105)), requires association with distinct LC3/GABARAP family proteins. This is, at least in part, dependent on ATG3 and ATG12-ATG5-ATG16(L) complex cooperation. Recently, the amino-terminal amphipathic helix in ATG3 has been annotated to sense the membrane curvature (95) and enables ATG3 to associate with highly curved membranes such as the tips of the growing phagophore. Remarkably, the PE-conjugation of GABARAP family proteins by ATG3 increases with the degree of membrane curvature. This allows incorporation of GABARAP family proteins at the phagophore tips and localization of GABARAPL2 at the site of closure (17). This is also illustrated in low-diameter, highly curved liposomes (50-200nm). Here the efficiency in PE-conjugation of

GABARAPL1, GABARAPL2 and LC3B is different (95). GABARAPL1 protein content is almost completely PE-conjugated at <100nm diameter membranes, whereas this is less effective for GABARAPL2 and LC3B (95). Since classical mammalian autophagosomes are approximately 500-1500nm in size (106), this suggests that GABARAPL1 can be efficiently incorporated in membranes of smaller-sized vesicles (95). The conjugation of GABARAPL1 is highly efficient even in absence of the ATG12-ATG5-ATG16(L) complex, suggesting that for small vesicles the absence of the ATG12-ATG5-ATG16(L) complex would promote incorporation of GABARAPL1 over other family proteins.

Together, increasing evidence is emerging that the cell is able to orchestrate LC3/GABARAP family protein recruitment through the cooperation of ATG3 and the ATG12-ATG5-ATG16(L) complex. Yet, although in yeast an Atg8 binding motif has been identified in Atg12 (100), it remains unclear if this motif exists in mammalian ATG12 and, when exists, has different affinity towards the other LC3/GABARAP family proteins. Potentially, the requirement of this complex for PE-conjugation of LC3B, but not for GABARAPL1 provides the cell with sufficient tools to specifically incorporate the desired LC3/GABARAP family protein into the vesicle.

II. Transcriptional regulation of LC3/GABARAP family proteins

Recent reports indicate that transcriptional mechanisms play important roles in the regulation of autophagic activity (107). The diversity of transcription factors that are capable of regulating the expression of LC3/GABARAP family proteins may reflect the requirement for specific family protein expression in e.g. cargo selection or autophagic rate and may depend on environmental factors (metabolic condition, pathogen exposure etc) or may reflect tissue specific effects (table 2).

GATA1/FOXO: The zinc finger DNA binding protein family of GATA (globin transcription factor) transcription factors regulate transcription during development and cell differentiation. GATA binding protein 1 (GATA1) plays an important role in erythropoiesis by controlling the growth and division (proliferation) of immature red blood cells. During erythropoiesis, GATA1 mediates expression of *LC3A*, *LC3B*, *GABARAP*, *GABARAPL1* and *GABARAPL2* (108). These effects are, at least in part, mediated through a member of the evolutionary conserved forkhead box O (FOXO) family of transcription factors, FOXO3. During erythropoiesis depletion of FOXO3 reduces the transcriptional effect of GATA1 and decreases upregulation of *LC3B*, *GABARAPL1* and *GABARAPL2* (108). In skeletal muscle, FOXO3 is also capable of regulating LC3B and GABARAPL1 expression, whereas in cardiomyocytes, neurons and hepatocytes FOXO1 is also able to fulfill a similar function (109-113).

UPR/ATF4/CHOP: Transcriptional upregulation of LC3/GABARAP family proteins may allow sustained autophagy activity through replenishment of the, during autophagy, degraded protein. For example, the high lysosomal turnover of LC3B during hypoxia-

induced autophagy in the absence of transcriptional replenishment results in LC3B depletion. Activation of the unfolded protein response (UPR) and production of activating transcription factor 4 (ATF4) (3) results in *LC3B* transcription and supports prolonged autophagy(3). In addition, ATF4 controls expression of *LC3A*, *GABARAPL1* and *GABARAPL2* in skeletal muscle-, cancer- and embryonic cells respectively (101,114,115). ATF4 induces expression of another transcription factor, CHOP/GADD153/DDIT3/C/EBP-homologues protein, which in turn controls *GABARAP* expression in embryonic cells (114).

Other regulatory mechanisms: Several other transcription factors have been shown to be able to regulate expression of *LC3/GABARAP* family proteins. Most identified factors are capable of increasing expression of the target gene, but also repressive factors have been identified, indicating the importance of gene expression regulation in autophagy. Transcription factor EB (TFEB), a member of the basic helix-loop-helix Leu zipper family has been identified as a key regulating transcription factor for autophagy related genes (107), including LC3B and LC3A (116,117). The extent of gene induction is controlled by the repressive transcription factor zinc-finger protein with KRAB and SCAN domain 3 (ZKSCAN3) (118). During starvation TFEB and ZKSCAN3 are regulated oppositely. Reduction of ZKSCAN expression results in increased *LC3B* and *GABARAPL2* mRNA expression (118). Whether the repressive effect of ZKSCAN3 on autophagy related genes is exclusively counteracting TFEB-mediated transcription, or that ZKSCAN3 opposes the general transcription of a set of autophagy-related genes, independent of transcription factor, remains elusive.

LC3B is the most studied and therefore best understood member of the *LC3/GABARAP* family. So far, 8 transcription factors have been identified that are capable of regulating its (basal) expression. In addition to the transcription factors mentioned, *LC3B* expression is controlled by CCAAT/enhancer binding protein (119), E2F1(120), c-jun (121,122) and sterol regulatory element binding protein 2 (SREBP2) (123). The diversity of stimulatory factors driving *LC3B* expression potentially emphasizes the essential role for LC3B in the execution of autophagy. Also for *GABARAPL1* many (6) transcription factors have been identified. *GABARAPL1* is induced in response to the transcription factors ATF4(101), GATA1(108), C/EBP β (119), FOXO1 (109,112), FOXO3 (108,111,112), and Estrogen receptor activation (124). Furthermore, *GABARAP* expression is controlled by GATA1 (108), CHOP (114), p38 α (125) and p73 (126) transcriptional activity.

To date no specific transcription factors have been found that regulate LC3C transcription. In clear cell renal cell carcinomas (ccRCC) expression of the tumour suppressor, von Hippel Lindau (VHL) protein, is frequently lost. Loss in VHL, amongst other effects, results in stabilization of hypoxia inducible factor (HIF) proteins. Interestingly, the loss of VHL results in repression of LC3C mRNA and protein expression. HIF2 α knock-down results in elevated expression of LC3C in VHL-deficient cells, illustrating the regulation of LC3C expression by VHL-activity through controlling HIF2 α stability (75).

Table 2: LC3/GABARAP family proteins transcription factors

LC3/GABARAP family member	Transcription factor/mechanism	Cell type/mechanism	Reference
LC3A	ATF4	Skeletal muscle	(115)
	TFEB	Lung cancer (A549 specifically)	(116)
	GATA1	Erythropoiesis	(108)
LC3B	ATF4	Colorectal cancer, glioblastoma, breast cancer	(3)
	C/EBP β	Liver (circadian rhythm)	(119)
	E2F1	Osteosarcoma	(120)
	FOXO1	Neurons, hepatocytes	(109,113)
	FOXO3	Skeletal muscle, erythropoiesis	(108,110)
	GATA1	Erythropoiesis	(108)
	c-Jun	Nasopharyngeal-, hepatocellular carcinoma	(121,122)
	SREBP2	Human Embryonic kidney	(117)
	TFEB	Cervical cancer	(118)
	ZKSCAN3 (repressive)	Various cancer cell lines	
LC3C	HIF2 α (repressive)	Clear cell renal cell carcinoma	(75)
GABARAP	CHOP	Mouse embryonic fibroblasts	(114)
	p38 α	Colorectal cancer	(125)
	GATA1	Erythropoiesis	(108)
	p73	Rhabdomyosarcoma	(126)
GABARAPL1	ATF4	Colorectal cancer, glioblastoma	(101)
	C/EBP β	Liver (circadian rhythm)	(119)
	Estrogen-regulated	Glandular Epithelial Cells	(124)
	FOXO1	Cardiomyocytes, hepatocytes	(109,112)
	FOXO3	Cardiomyocytes, skeletal muscle, Erythropoiesis	(108,111,112)
	GATA1	Erythropoiesis	(108)
GABARAPL2	ATF4	Mouse embryonic fibroblasts	(114)
	GATA1	Erythropoiesis	(108)
	ZKSCAN3 (repressive)	Bladder carcinoma	(118)

III. Post-translational modification

The best understood post-translational modification of the LC3/GABARAP family protein is the conjugation to PE. This event determines the availability for the family protein to actively participate in autophagy or its related processes. Other post-translational modifications of LC3/GABARAP family proteins are still poorly understood.

Phosphorylation: LC3B phosphorylation by Serine/threonine-protein kinase 3 (STK3) and Serine/threonine-protein kinase 4 (STK4) is important in lysosomal fusion. Failure to phosphorylate LC3B-Thr50 results in failure of fusion of autophagosomes with lysosomes and inhibition of autophagic flux (127). Interestingly, Thr50 is conserved in LC3A-a and -b and LC3C, but is absent in the GABARAP family proteins (Fig. 2). In addition, Protein Kinase A (PKA) phosphorylates murine LC3B at serine 12 which restrains LC3B conjugation and autophagosome association. During nutrient depleted conditions PKA activity decreases, leading to rapid activation and availability of LC3B for the execution

of autophagy (128). Thus, PKA suppresses autophagy and allows existence of a phosphorylated, unconjugated LC3B pool. This pool is then readily available for rapid induction of autophagy. In humans, the 12th amino acid of LC3B is a threonine. Yet, PKA is capable of phosphorylating both serine and threonine residues (129). In LC3A-a, LC3C and GABARAPL2 a serine is observed at position 12 (Fig. 2). While this suggests that the post-translational phosphorylation status of LC3B can regulate the size of the protein pool available for conjugation, it remains unknown, however plausible, if these events regulate other LC3/GABARAP family proteins.

Acetylation: LC3B cycles between nucleus and cytoplasm a process which is regulated by acetylation of LC3B at Lys49 and Lys51 which prevents LC3B from entering the cytoplasm and interaction with ATG7 for further processing. Nutrient deprivation results in deacetylation of nuclear LC3B by sirtuin 1 (SIRT1) leading to an increased cytoplasmic translocation and available substrate for ATG7 (130). Interestingly, both lysine residues at 49 and 51 are present in all LC3/GABARAP family proteins, suggesting a common regulatory mechanism for availability. These insights suggest that post-translational modifications are an important system for regulation LC3B availability and rapid induction of autophagy. If this type of regulation is important for the other LC3/GABARAP family proteins remains to be investigated.

MAP1LC3B	-----MPSEKTFKQRR Y FEQ [*] RVEDVRLIREQHPTKIPVIERKYGEKQLPVLD Y TKFLV	54
MAP1LC3A-a	-----MPSDRPFKQRR S FADRCKEVQIQIRDQHPSKIPVIERKYGEKQLPVLD Y TKFLV	54
MAP1LC3A-b	--MKMRFFSSPCGKA [*] AVDPADRCKEVQIQIRDQHPSKIPVIERKYGEKQLPVLD Y TKFLV	58
MAP1LC3C	MPPFPQKIPSVRPFKQ R S LAIRQEEVAGIRAKFPNKIPVVVERYPRETFLPPLD Y TKFLV	60
GABARAP	-----MKFVYKEEHPFEKRRSEGEKIRKKYPDRVVPVIVEKAPKA-RIGDLD Y KKYL	51
GABARAPL1	-----MKFQYKEDHPFEYRKKEGEKIRKKYPDRVVPVIVEKAPKA-RVPDLD Y KKYL	51
GABARAPL2	-----MKWMPFKEDH S LEHRCVESAKIRAKYPDRVVPVIVEKVS [*] GS-QIVDID Y KKYL	51
	* * ** * ** *	
MAP1LC3B	PDHVNMS [*] ELIKIIRRLQLNANQAFFLLVNGHSMVSVSTPISEVYSEKDEDEGFLYMVYA	114
MAP1LC3A-a	PDHVNMS [*] ELVKIIRRLQLNPTQAFLLVNQHSMVSVSTPIADIYEQEKDEDEGFLYMVYA	114
MAP1LC3A-b	PDHVNMS [*] ELVKIIRRLQLNPTQAFLLVNQHSMVSVSTPIADIYEQEKDEDEGFLYMVYA	118
MAP1LC3C	PQELTMTQFLSIIRSRMVLRA [*] TEAFYLLVNNKSLVMSATMAEIIYRDYKDEDEGFLYMTYA	120
GABARAP	PSDLTVGQFYFLIRKRIHLRAEDALFFVNNV-IPPTSATMGQLYQEHEEDFFLYIAYS	110
GABARAPL1	PSDLTVGQFYFLIRKRIHLRPEDALFFVNNV-IPPTSATMGQLYEDNHEEDFFLYVAYS	110
GABARAPL2	PSDITVAQFMWIIIRKRIQLPSEKAI [*] FLFVDKT-VPQSSLTMGQLYEKDEDEGFLYVAYS	110
	** * * * * * *	
MAP1LC3B	SQET F GMKLSV-----	125
MAP1LC3A-a	SQET F GF-----	121
MAP1LC3A-b	SQET F GF-----	125
MAP1LC3C	SQET F GCLESAA [*] PRDGSSLED [*] RPCNPL	147
GABARAP	DESVYGL-----	117
GABARAPL1	DESVYCK-----	117
GABARAPL2	GENT F GF-----	117
	*	

Figure 2. LC3/GABARAP family protein alignment.

The alignment of human LC3/GABARAP displays 27 conserved amino acids in for all proteins (*). This includes the glycine (grey) for PE –conjugation. Potential phosphorylation (yellow) and acetylation (green) events are indicated.

Functional variance by differential (affinity in) binding partners between LC3/GABARAP family proteins

Besides similarities, functional differences are attributed to LC3/GABARAP family proteins. Important in establishing these differences are the interactions with binding partners. Interestingly, extensive proteomics analysis of binding partners of LC3/GABARAP family proteins has demonstrated that there is overlap in interactome; however, more importantly, that there are clear differences in binding partners. These data are generated based on pull-down assays and will be vital in identifying differences in affinity, specificity in function and biological relevance (37).

The difference in interactome for the LC3/GABARAP family proteins may depend on differential properties of the LC3/GABARAP family proteins. The structure of the LC3/GABARAP family proteins comprises a carboxyl-terminus ubiquitin-like (UBL) core (a conserved ubiquitin fold) and two amino-terminal helices. Binding of the LIR to the LC3/GABARAP family protein occurs at the interface of the amino-terminal arm and the UBL core. The surface of helix 1 of LC3 is basic compared to the acidic nature of GABARAPL2 and GABARAP. The surface of helix 2 is acidic, neutral and basic for respectively LC3, GABARAPL2 and GABARAP. The difference in helices 1 and 2 electrostatic surface potential in LC3/GABARAP family proteins together with differences in the LIR in autophagy receptors contributes to variation in binding partners (10). This is also illustrated by the total number of interacting proteins, of which approximately one-third are specific for LC3 subfamily, one-third specific for GABARAP subfamily and one-third is associated with both (37). An interesting example in this context is LC3C. According to proteomic analysis, LC3C has the least overlapping interactors. Properties of LC3C and its unique interacting partner NDP52 have established its indispensable function in xenophagy.

This emphasizes that subtle differences between proteins allow specific interactions and illustrates the complexity of their functions.

Closing remarks

There are at least seven orthologues of yeast Atg8 known in humans. Despite the high structural similarities between the LC3/GABARAP family proteins, the existing differences in LIR, amino terminal helices and conformation that makes each family protein unique in binding partners and function. Valuable data on e.g. pull-down assays which contribute to mapping the autophagy interaction network (37) have provided important insights in the interactome. Their roles in autophagy and specific sequestration of cargo are diverse. How the cell orchestrates this complex recruitment to fulfill these specific functions is highlighted in this review. Differences in conjugation (stress-induced regulation; dependent on vesicle curvature), binding partners and expression (*via* differential

transcriptional regulation) and post-translational regulation ((de)phosphorylation and (de)acetylation events), contribute to the systems complexity. Increasing evidence indicates that the LC3/GABARAP proteins display functions that are unrelated to canonical autophagy. This has important implications for functional experiments using knockdown models or factors that influence expression of the family proteins. Unravelling the role of the individual LC3/GABARAP family proteins is important to distinguish specialized functions attributed to each specific family protein for autophagy (un)related functions.

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Chapter 3

GABARAPL1 is required for increased EGFR membrane expression during hypoxia

Tom G. Keulers^{1*}, Marco B.E. Schaaf^{1*}, Hanneke J.M. Peeters¹, Kim G.M. Savelkouds¹,
Marc A. Vooijs¹, Johan Bussink², Barry Jutten¹, Kasper M.A. Rouschop¹

¹Maastricht Radiation Oncology (MaastRO) lab, GROW – School for Oncology and
Developmental Biology, Maastricht University, Maastricht, the Netherlands

²Department of Radiation Oncology, Radboud University Medical Center, Nijmegen,
the Netherlands

*contributed equally

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Abstract

Background and purpose The epidermal growth factor receptor (EGFR) is overexpressed, amplified or mutated in various human epithelial tumours and hypoxia is a common feature of solid tumours. Both EGFR and hypoxia are associated with therapy resistance and poor treatment outcome. To survive hypoxia, cells adapt by activation of hypoxia responsive pathways and expression of hypoxia-induced plasma membrane proteins. We observed that GABA_A receptor associated protein like1 (GABARAPL1) and plasma membrane expression of EGFR were increased during hypoxia. Here we explored the role of the GABARAPL1 in EGFR membrane expression during hypoxia.

Material and methods Quantitative qPCR, immunoblot analysis, flow cytometry and cytochemistry were used to assess this interplay.

Results GABARAPL1 mRNA and protein levels are increased during hypoxia *in vitro* and correlate with tumour hypoxia in a panel of primary HNSCC xenografts. High GABARAPL1 mRNA is associated with poor outcome of HNSCC patients. During hypoxia, EGFR membrane expression is increased and GABARAPL1 and EGFR colocalize at the plasma membrane. GABARAPL1 knockdown inhibits EGFR membrane expression during hypoxia.

Conclusion GABARAPL1 is required for increased membrane expression of EGFR during hypoxia, suggesting a role for GABARAPL1 in the trafficking of these membrane proteins.

Introduction

Solid tumours are characterized by the presence of abnormal and immature vasculature that leads to extreme homogeneities in oxygenation. Oxygenation in these regions varies from normal (approx. 5% O₂) to severe hypoxia and even anoxia [1, 2]. Tumour cells within hypoxic regions contribute to increased invasion [3], metastatic spread [4] and therapy resistance [5].

For survival, cancer cells adapt to hypoxia by activation of several signalling pathways including the hypoxia inducible factor (HIF) pathway and the unfolded protein response (UPR). During normoxic conditions, the HIF-1 α protein is ubiquitinated and rapidly degraded. HIF1 α is stabilized during hypoxia and modifies the microenvironment by upregulating expression of several proteins. For example, through increasing vascular endothelial growth factor (VEGF) expression and induction of angiogenesis [6]. Other HIF1 transcriptional targets are carbonic anhydrase 9 (CA9) and Glucose Transporter 1 (GLUT1). These proteins are respectively involved in deacidification and increased glucose uptake during hypoxia [7].

The UPR is activated during hypoxia as a consequence of increased misfolded or unfolded proteins [8]. The UPR constitutes of three parallel signalling cascades initiated by PERK (PKR-like ER kinase), IRE1 (inositol-requiring protein 1) and ATF6 (activating transcription factor 6). PERK is a kinase that phosphorylates eIF2 α to inhibit translation initiation. The blockade in overall protein translation allows selective translation of the transcription factor, ATF4 [9], and subsequently CHOP [10]. The UPR is important for inhibition of mRNA translation [11], promote ER homeostasis [12], increasing the cell's antioxidant capacity [13] and induce expression of several autophagy-related genes [14] [15] during hypoxia.

Epidermal growth factor receptor (EGFR) signalling is increased in many cancer types due to overexpression, amplification or mutation. Activation of the receptor leads to a cascade of signalling pathways that control growth, migration and differentiation and acquisition of an EMT-like phenotype [16]. EGFR is upregulated during hypoxia [17] and combined targeting of EGFR and hypoxia modification is expected to increase tumour responsiveness to radiotherapy [18].

After production, membrane proteins, including EGFR, require active transportation to the plasma membrane. GABARAPL1 is a member of the GABARAP protein family, which consists of 3 members, GABARAP, GABARAPL1 and gate-16/GABARAPL2. Although their function is largely unknown, they are implicated in several vesicular mechanisms as autophagy and receptor trafficking. For example, GABARAPL1 promotes cell surface expression of the Kappa Opioid Receptor (KOR) by facilitating its trafficking along the secretory pathway. Accordingly, knockdown of GABARAPL1 decreases the surface expression of KOR [19]. Binding of GABARAPL1 to the KOR is based on hydrophobic interactions, and the N-terminal region of GABARAPL1 is important for binding

to tubulin and surface expression of the KOR [20]. In addition, the tubulin binding region is important for GABA_A receptor clustering [21].

We observed that EGFR membrane expression and GABARAPL1 production are increased during hypoxia. We therefore hypothesized that GABARAPL1 mediates increased EGFR membrane-expression.

Material and methods

Cell culture Colorectal adenocarcinoma (HT29, HCT116), mammary adenocarcinoma (MCF7), glioblastoma astrocytoma (U87, U373) cells and large cell lung cancer (H460) were maintained as described by ATCC. Doxycycline-inducible (1µg/ml) shRNA targeting GABARAPL1 [TTACCTTACTTCATACTTGCCC] or scrambled control [CGAGGGCGACTTAACCTTAGG] was achieved through lentiviral pTripZ (Thermo Scientific) expression as described previously [14]. Knockdown of UPR components (siRNAs) and HIF1α (shRNA expression) was done as described previously [15]. Hypoxia exposure was done using a MACS VA500 workstation (Don Whitley Scientific) that allows maintaining a stable O₂ concentration over time due to continuous gas influx.

Immunoblot Cells were lysed and processed as described previously [11]. Proteins were probed with antibodies against GABARAPL1 (Proteintech group, 11010-1-AP), EGFR (SC03, Santa Cruz), eIF4E, p-ERK (Cell signaling) and Beta-Actin (MP Biomedicals). Bound antibodies were visualized using HRP-linked antibodies (Cell Signaling).

Membrane isolation Cells were exposed to anoxia (O₂< 0.02 %), trypsinized and resuspended in hypotonic swelling buffer (10mM Tris, 1mM β-mercaptoethanol, 100 µM EDTA and HEPES, pH 7.3) for 1h on ice. Cell ghosts were ultracentrifuged at 145000xg for 30 minutes and analysed by western blot.

Clinical study GABARAPL1 mRNA expression was determined by quantitative PCR in 86 head and neck cancer patients [22]) and used to median-dichotomize the patient cohort. Differences between patient characteristics (Suppl table1) were assessed with a χ^2 -test or student's t-test. Differences in recurrence-free survival were determined using Kaplan-Meier survival analysis and Log-Rank (Mantel-Cox) test.

Immunofluorescence Cells were fixed with 4% paraformaldehyde, permeabilized, blocked and probed with anti-GABARAPL1 (Aviva Systems Biology, ARP55398_P050) and anti-EGFR (Oregon Green-labeled Cetuximab). Anti-GABARAPL1 was visualized using goat anti-rabbit Alexa-555 (Invitrogen, A-21428).

Quantitative real-time PCR mRNA was isolated with NucleoSpin RNA II kit (Bioke) and reverse transcribed using I-script (BIO-RAD). Expression was normalized to 18S. Primers used are listed in Supplementary table 2.

Flow cytometry single cells were labelled with anti-EGFR (Cetuximab-oregon green) or anti-CA9-hiLyte-488(Novus Biologicals) and anti-GLUT1-APC(BD biosciences) Membrane expression was determined by flow cytometry..

Click-it chemistry: After 16 hours of anoxia exposure, azide-labeled methionine (L-AHA) was allowed to incorporate for 8 hours. Subsequently, cells were trypsinised and labelled by click-it chemistry according to the manual (Thermo Fisher) and analysed by flow cytometry.

Statistics Data were analysed using GraphPad Prism. Student's *t* test was used for single comparisons. Multiple testing was done using a repeated measures ANOVA with a Bonferroni post-hoc test. *P*-values < 0.05 were considered statistically significant.

Results

GABARAPL1 expression is increased during hypoxia

Previously, microarray analysis on HT29, MCF7 and DU145 cancer cell lines during hypoxia ($O_2 < 0.02\%$) were performed [23]. In all three cell lines, hypoxia increased GABARAPL1 mRNA expression (Suppl. Fig. 1A). These results were validated on independent experiments by qPCR analysis of HT29, MCF7, DU145, U87 (Fig. 1A), U373 (fig1B) and HCT116 (fig1C) cells. GABARAPL1 induction is dependent on severity of hypoxia as moderate hypoxia ($0.2\% O_2$) is less capable of inducing GABARAPL1 expression in HT29 and U373 cells (Suppl. Fig 1B) after 24 hours exposure. In line with RNA expression, GABARAPL1 protein expression levels are induced during anoxia ($O_2 < 0.02\%$) in U87 and HT29 cells, but not in U373 (Fig. 1D).

Cells adapt to hypoxia through activation of multiple pathways, including HIF and UPR signalling. We therefore investigated the contribution of these pathways to hypoxia-induced GABARAPL1 expression. Knockdown of HIF1 α did not change the extent of GABARAPL1 upregulation in HT29 and U373 cells during hypoxia (Fig. 1C). Next, we investigated the three parallel arms of the UPR; PERK, ATF6 and IRE1. Knockdown of PERK, but not ATF6 or IRE1 abrogated hypoxia-induced GABARAPL1 mRNA expression (Fig. 1D). Downstream of PERK, targeting ATF4 or CHOP revealed that ATF4 is required for GABARAPL1 mRNA upregulation during hypoxia. Together, this indicates that transcriptional upregulation of GABARAPL1 during hypoxia is mediated by PERK/ATF4 signalling.

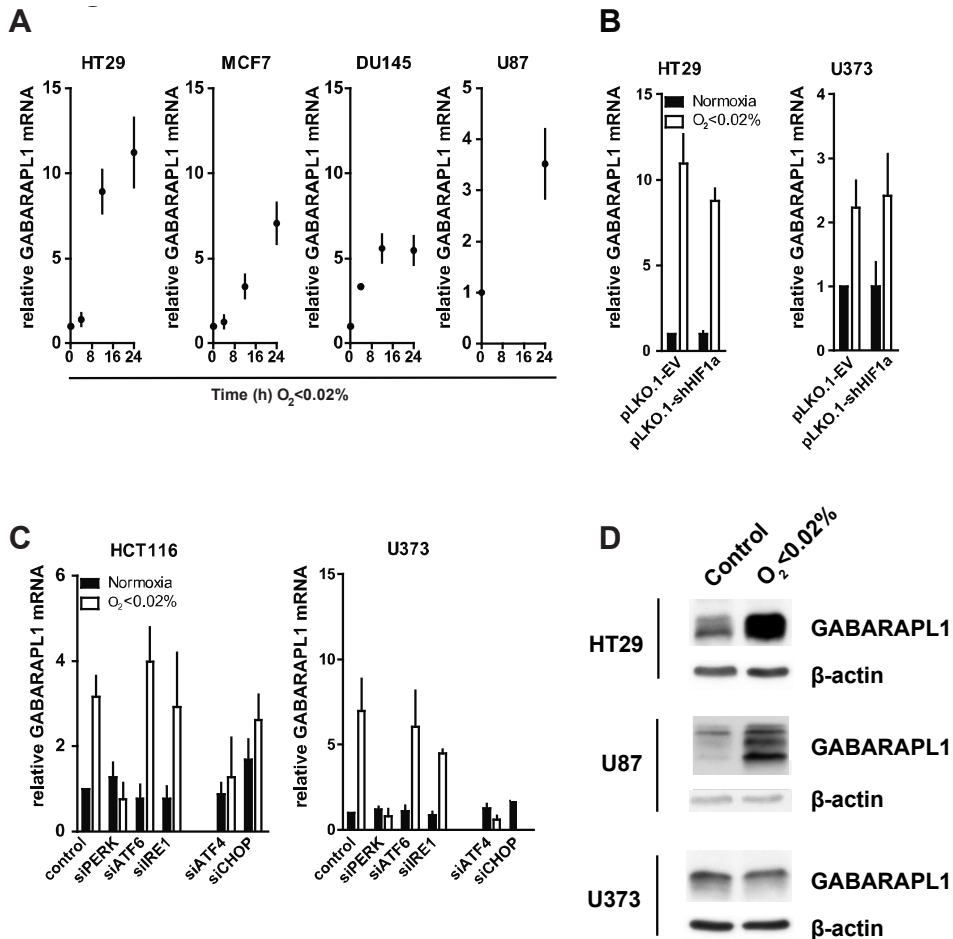


Figure 1. Increased GABARAPL1 expression is PERK/ATF4 dependent.

(A) Transcriptional regulation of GABARAPL1 in HT29, MCF7, Du145 and U87 cancer cells during anoxia ($O_2 < 0.02\%$). (B) HIF1 α knockdown (shHIF1 α) in HT29 and U373 cells does not affect GABARAPL1 mRNA expression. (C) Transcriptional upregulation of GABARAPL1 during hypoxia is dependent on PERK/ATF4 signaling. A-C, $n=3$, mean \pm SEM. (D) GABARAPL1 protein expression in induced during anoxia ($O_2 < 0.02\%$)

GABARAPL1 mRNA expression correlates with tumour hypoxia and poor clinical outcome

As GABARAPL1 is upregulated during hypoxia, we assessed whether GABARAPL1 mRNA expression could serve as surrogate marker for tumour hypoxia. In a panel of 14 primary human head and neck squamous cell carcinoma (HNSCC) xenografts [24], GABARAPL1 mRNA expression and hypoxia (pimonidazole) were determined. The hypoxic fraction [25] correlated with GABARAPL1 mRNA expression (Fig. 2A; Pearson $r=0.63$; $p<0.05$), suggesting that GABARAPL1 mRNA can be used as a surrogate marker for tumour hy-

poxia in HNSCC. In agreement with the PERK/ATF4-dependence, GABARAPL1 mRNA expression correlates with another UPR-dependent hypoxia regulated gene, LC3b [15](Fig. 2B; Pearson $r=0.54$; $p<0.05$).

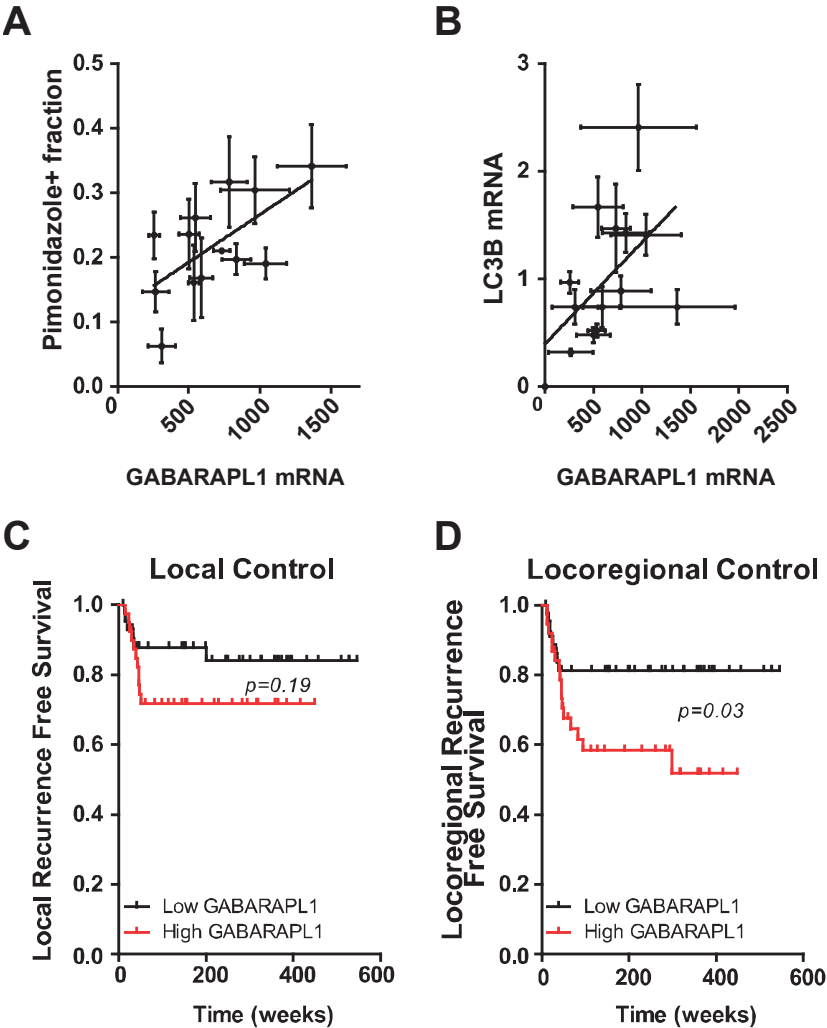


Figure 2. GABARAPL1 mRNA expression correlates with tumour hypoxia and poor clinical outcome.

(A) In 14 HNSCC xenografts, GABARAPL1 mRNA expression was determined and correlated with (hypoxic fraction) pimonidazole) and (B) LC3b mRNA. (C) high GABARAPL1 mRNA expression is associated with increased local ($p=0.10$) and (D) locoregional recurrences ($p=0.03$) in a cohort of 86 HNSCC patients.

Tumour hypoxia is a negative prognostic and predictive factor of patient outcome as it decreases the efficacy of radio- and chemotherapy. This has been particularly well established in HNSCC [26, 27].

We therefore questioned if GABARAPL1 expression has prognostic value in a cohort of 86 HNSCC patients after chemoradiation [22]. Local ($p=0.19$) and locoregional ($p=0.038$) control was worse in patients with high GABARAPL1 expressing tumours (Fig. 2C and 2D)

GABARAPL1 is required for increased EGFR membrane expression during hypoxia

Recent reports indicate that EGFR expression is increased during hypoxia [17]. To investigate if GABARAPL1 mediates EGFR membrane expression, cellular localization was assessed using confocal microscopy. In U87 cells, GABARAPL1 is present in a typical vesicle-like pattern. During anoxia, GABARAPL1 is also observed at the cellular perimeter and colocalizes with EGFR at the plasma membrane (fig 3A). In accordance, both EGFR and GABARAPL1 are enriched in the membrane fraction of anoxia exposed HT29 cells (Fig. 3B).

To determine if GABARAPL1 is involved EGFR membrane-expression, doxycycline-inducible GABARAPL1 knockdown cells were engineered (Fig 3C). EGFR expression at the plasma membrane of control and GABARAPL1 knockdown in HT29, U373, MDA-MB231 and U87 cells after exposure to hypoxia (O_2 0.2%) and anoxia ($O_2 < 0.02$ %) was determined by flow cytometry.

EGFR membrane expression is induced in all control (SCR) cell lines during hypoxia and anoxia (Fig 3D). This increased EGFR membrane expression was inhibited after GABARAPL1 knockdown (Fig 3D)

To determine if the reduced EGFR membrane expression is the result of a reduced total EGFR expression, EGFR mRNA and total protein expression was determined. During hypoxic and anoxic conditions, EGFR mRNA and total protein levels are not altered or even increased in GABARAPL1 knockdown cells (Fig 3F). Notably, EGFR mRNA of U373 and MDA-MB-231 GABARAPL1 knockdown cells during normoxic conditions was increased (FIG 3E).

To determine if GABARAPL1 is required uniquely for EGFR trafficking during hypoxia, overall membrane protein expression in SCR control and GABARAPL1 knockdown cells was determined using *click-it* chemistry. GABARAPL1 knockdown reduces membrane protein expression in both HT29 (23%) and MCF7 (43%), indicating a role in protein trafficking broader than EGFR (Fig 3G). In line, CA9 plasma membrane expression was decreased after GABARAPL1 knockdown in 4 cell lines tested (Suppl fig 2A). Reduced CA9 membrane expression was not caused by reduced production in GABARAPL1 knockdown cells as indicated by total protein expression (Fig 3F) and qPCR (Suppl. Fig 2C). Interestingly, in contrast to EGFR and CA9, GLUT1 membrane expression is not affected by GABARAPL1 knockdown, despite increased CA9 and GLUT mRNA expression in MDA-MD-231 GABARAPL1 knockdown cells (Suppl Fig 2C and 2D).

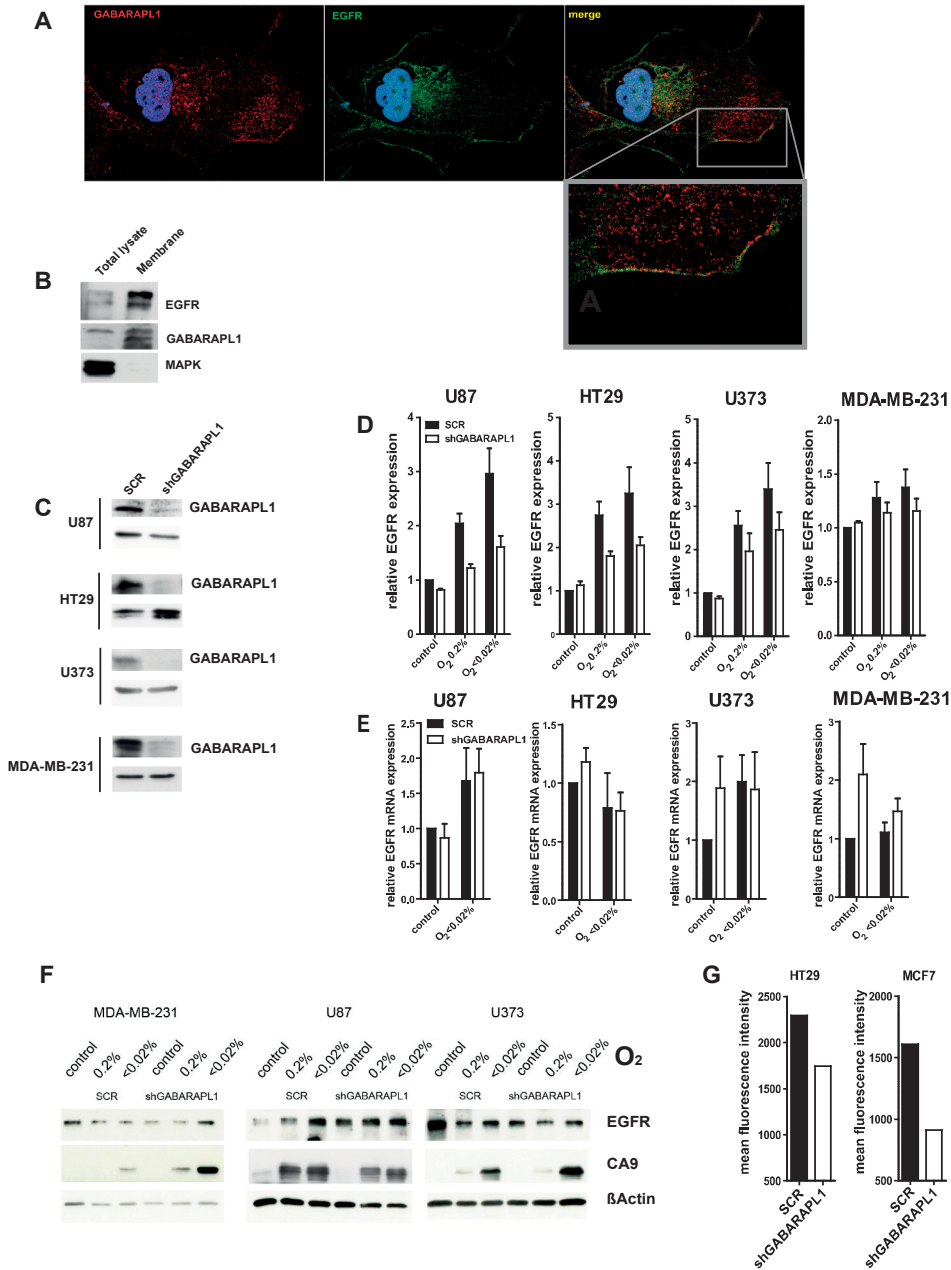


Figure 3. GABARAPL1 is required for increased EGFR membrane expression during hypoxia.

(A) Immunofluorescent staining of GABARAPL1(red) and EGFR(green) in U87 cells. (B) immunoblot of total and membrane fraction of anoxic HT29 cells. (C) GABARAPL1 knockdown cell lines. (D) FACS analysis of EGFR membrane expression during hypoxic (O₂ 0.2 %), and anoxic (O₂ <0.02 %), conditions n=4 mean±SEM (E) EGFR mRNA expression. n=3 mean±SEM (F) Immunoblot of total EGFR and CA9 levels (G) Membrane expression of proteins assessed by Click-it chemistry.

Discussion

EGFR expression and tumour hypoxia are negative prognostic factors associated with poor treatment outcome. In this study we explored the role of GABARAPL1, which we identified as a hypoxia induced protein required for the trafficking of EGFR to the plasma membrane during hypoxia.

Here we show that the GABARAP family member GABARAPL1 is induced during hypoxia on both mRNA and protein levels. Interestingly, other GABARAP family members, GABARAP and GABARAPL2, are not induced during hypoxia, suggesting a differential role for gabarapl1 during these conditions (data not shown). Several proteins, including EGFR can be upregulated during hypoxia [17] and require transportation to the membrane. Since the GABARAP family is implicated in several processes like anterograde trafficking of several receptors like the Kappa Opioid Receptor [19], angiotensin [28, 29] and GABA_A receptor [30] we questioned the role of GABARAPL1 in EGFR trafficking. EGFR and other growth factor receptors are often amplified or mutated in cancer and may promote hypoxia tolerance [16, 31]. In this report we show that the membrane expression of EGFR is elevated during hypoxia and that this effect is GABARAPL1 dependent. It is therefore tempting to speculate that the observed relation between high GABARAPL1 and outcome is partly mediated through facilitating enhanced EGFR-signalling.

Hypoxia induced membrane expression of EGFR in most of the cell lines tested. Although consistent, this was less pronounced in the parental cell lines compared to the genetically modified cell lines (Suppl Fig 3). In the pTRIPZ lentiviral system, parallel to shRNA expression, red fluorescent protein is expressed. This may lead to increased misfolding of proteins and/or ROS formation and may contribute to increased EGFR upregulation during hypoxia in the transgenic cells.

For efficient membrane expression and further downstream signalling, clustering of receptors, including EGFR is essential. Chen et al showed that the tubulin-binding motif of GABARAP is required for clustering of the GABA_A receptor [32]. Also GABARAPL1 contains this binding motif, which is located in the first 22 amino acids [33]. GABARAPL1 acts as a linker between microtubules and the KOR to facilitate anterograde trafficking [34]. GABARAPL1 may act similarly in EGFR receptor clustering and/ or trafficking.

Together our data support the specific expression and/or translocation of proteins during hypoxia that allow cells to adapt to conditions of low oxygenation. GABARAPL1 in this respect seems to fulfil a specific role in trafficking of EGFR and CA9.

Acknowledgements

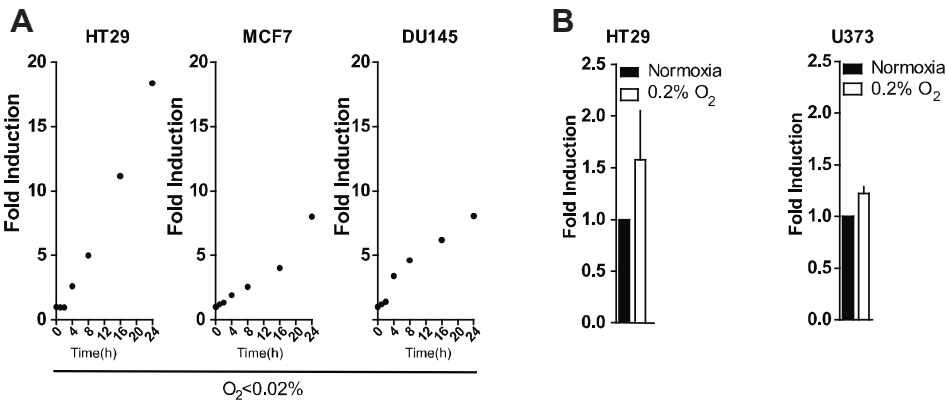
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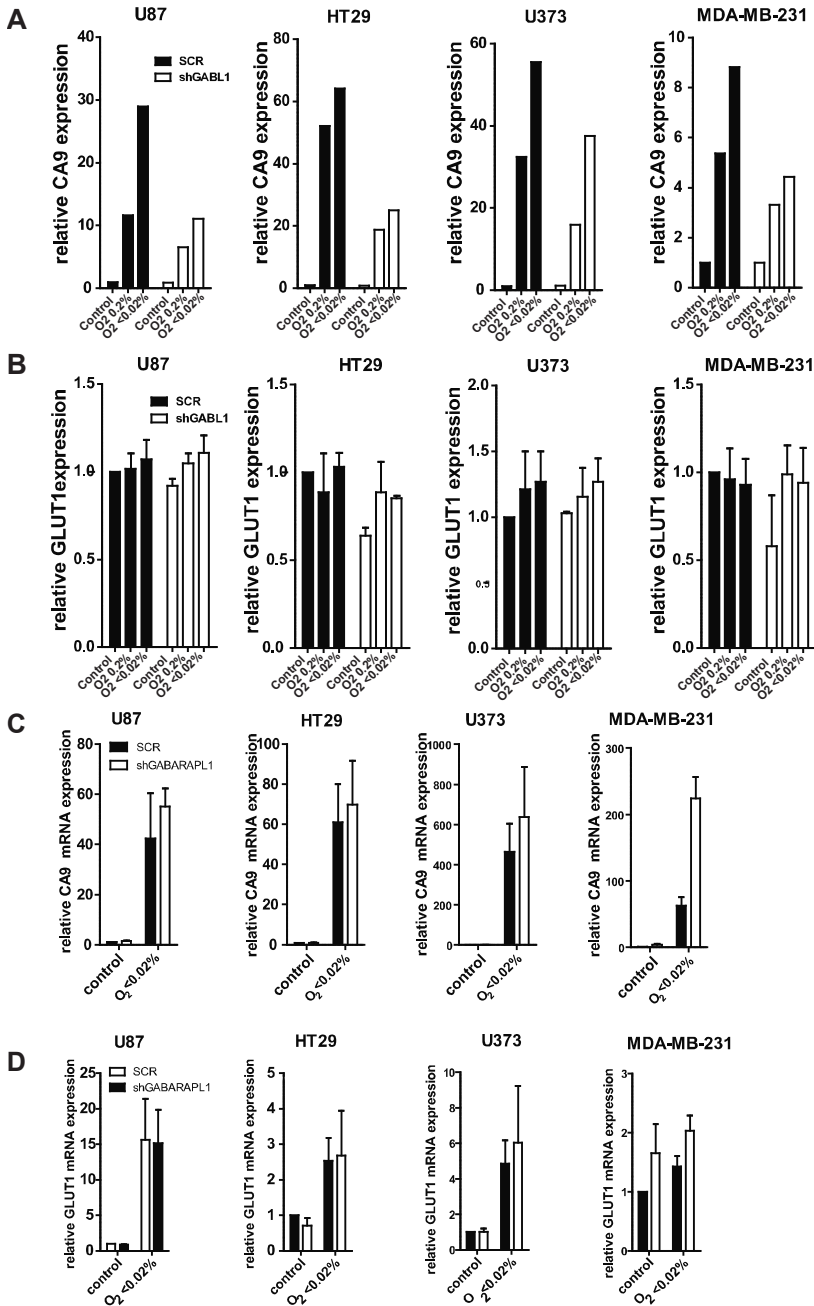
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Supplementary figures



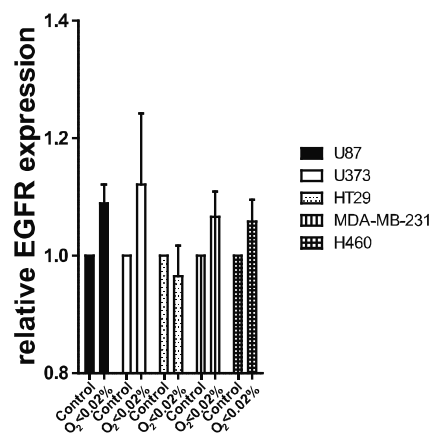
Supplementary Figure 1

- A) Micro array analysis shows an increased GABARAPL1 expression during hypoxia ($O_2 < 0.02\%$)
- B) GABARAPL1 mRNA expression during hypoxia ($O_2 0.2\%$) as assessed by qPCR $n=3$ mean \pm SEM



Supplementary figure 2

(A B) Membrane expression levels of CA9(A) and GLUT1(B) as determined by FACS in GABARAPL1 knockdown cells during hypoxia (0.2% O₂) and anoxia (<0.02% O₂). Shown graph is a representative example n=3. B) n=3 mean ±SEM (C D) relative mRNA expression of CA9(C) and GLUT1(D) in GABARAPL1 knockdown cells during anoxia(<0.02% O₂) n=4 mean±SEM



Supplementary figure 3

Relative EGFR expression in parental cell lines as determined by FACS n=3 mean± SEM

Chapter 4

EGFRvIII expression triggers a metabolic dependency and therapeutic vulnerability sensitive to autophagy inhibition

Barry Jutten¹, Tom GH Keulers¹, Hanneke JM Peeters¹, Marco BE Schaaf¹, Kim GM Savelkoul¹, Inge Compter¹, Ruud Clarijs², Olaf EMG Schijns³, Linda Ackermans³, Onno PM Teernstra³, Marijke I Zonneveld¹, Resi ME Colaris², Ludwig Dubois¹, Marc A Vooijs¹, Johan Bussink⁴, Julio Sotelo⁵, Jan Theys¹, Guido Lammering^{1,6} and Kasper MA Rouschop¹

¹Maastricht Radiation Oncology (MaastRO) lab, GROW – School for Oncology and Developmental Biology, Maastricht University, Maastricht, the Netherlands

²Department of Clinical Pathology, Zuyderland MC, Sittard-Geleen, The Netherlands

³Department of Neurosurgery, Maastricht University Medical Centre

⁴Department of Radiation Oncology, Radboud University Medical Center, Nijmegen, The Netherlands

⁵Neuroimmunology and Neuro-Oncology Unit, National Institute of Neurology and Neurosurgery, Mexico City, Mexico

⁶Heinrich-Heine University Düsseldorf, Germany

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Abstract

Expression of EGFRvIII is frequently observed in glioblastoma and is associated with increased cellular proliferation, enhanced tolerance to metabolic stresses, accelerated tumour growth, therapy resistance and poor prognosis. We observed that expression of EGFRvIII elevates the activation of macroautophagy/autophagy during starvation and hypoxia and explored the underlying mechanism and consequence. Autophagy was inhibited (genetically or pharmacologically) and its consequence for tolerance to metabolic stress and its therapeutic potential in (EGFRvIII⁺) glioblastoma was assessed in cellular systems, (patient derived) tumour xenografts and glioblastoma patients. Autophagy inhibition abrogated the enhanced proliferation and survival advantage of EGFRvIII⁺ cells during stress conditions, decreased tumour hypoxia and delayed tumour growth in EGFRvIII⁺ tumours. These effects can be attributed to the supporting role of autophagy in meeting the high metabolic demand of EGFRvIII⁺ cells. As hypoxic tumour cells greatly contribute to therapy resistance, autophagy inhibition revokes the radioresistant phenotype of EGFRvIII⁺ tumours in (patient derived) xenograft tumours. In line with these findings, retrospective analysis of glioblastoma patients indicated that chloroquine treatment improves survival of all glioblastoma patients, but patients with EGFRvIII⁺ glioblastoma benefited most. Our findings disclose the unique autophagy dependency of EGFRvIII⁺ glioblastoma as a therapeutic opportunity. Chloroquine treatment may therefore be considered as an additional treatment strategy for glioblastoma patients and can reverse the worse prognosis of patients with EGFRvIII⁺ glioblastoma.

Introduction

EGFR (epidermal growth factor receptor) is a transmembrane glycoprotein and 1 of 4 members of the ERBB family of tyrosine kinase receptors. Binding of one of its natural ligands results in homo- or heterodimerization of the receptor, autophosphorylation and subsequent activation of signal transduction pathways. These pathways are involved in regulating cellular proliferation, differentiation, and survival.(1,2) Although present in normal cells, EGFR is overexpressed in a variety of tumours and has been associated with poor prognosis and decreased survival (3). EGFR activation also plays a role in resistance to chemotherapy and radiation treatment in tumour cells (4,5).

In cancer, EGFR is often mutated, leading to enhanced or sustained receptor signaling. One of the most common variants of EGFR is an exon 2–7 deletion mutant, EGFRvIII (also termed Δ EGFR or de2-7EGFR) (6-9). This deletion results in a truncated receptor that lacks 267 amino acids in the extracellular binding domain. This deletion leads to important functional changes; the receptor is unable to bind its ligands, but becomes constitutively active, resulting in uncontrolled pro-oncogenic effects and thereby promotes neoplastic transformation and tumourigenicity (10-13). EGFRvIII expression is observed in several malignancies, including glioblastoma (GBM), non-small lung cell carcinoma, breast cancer, prostate cancer and head and neck cancer (6,8,9) and is associated with poor prognosis (14). In GBM patients with amplification of the *EGFR* gene, the overall prevalence of EGFRvIII is 50–60% (15) and has been reported to contribute to tumour stem cell maintenance (16-18). Expression of EGFRvIII greatly enhances GBM tumourigenicity *in vivo* (19,20) and stimulates cell invasion *in vitro* and *in vivo* (21,22).

The tumour microenvironment is characterized by extreme heterogeneities in nutrient supply and oxygenation that arise primarily due to a poorly developed and/or functioning vascular network. Gradients of oxygenation exist around individual perfused vessels and range from normal values ($\sim 5\% \text{ O}_2$) near the vessel wall to anoxia in perinecrotic regions. Transient changes in blood flow also lead to strong temporal changes in oxygenation within specific tumour regions (23). The percentage of viable hypoxic tumour cells within individual tumours with otherwise similar clinical features varies tremendously among patients (24) and is clinically important because high levels of tumour hypoxia correlate with poor prognosis and a more aggressive phenotype (24-26). The source of the variability in hypoxia among different tumours is likely due to the acquisition of changes that drive increased hypoxia tolerance (27). Strikingly, EGFRvIII expression *in vivo* is rapidly lost when tumour cells are cultured *in vitro* under nutrient-rich conditions (28). This observation suggests that in the tumour microenvironment, which is characterized by heterogeneities in nutrient supply and oxygenation, EGFRvIII-expressing tumour cells have a survival advantage, a finding supported by pre-clinical evidence (12,13).

To survive starvation and hypoxia, cells respond by upregulation of autophagy (Greek for “self-eating”) (29,30). A number of recent studies have demonstrated that

human tumour cell lines induce autophagy when exposed *in vitro* to hypoxia and/or metabolic stress, (31-33) which mediates both selective and bulk degradation of proteins, cytoplasmic content, and organelles, and enables a cell to recycle constituents and provide itself with the necessary nutrients to maintain energy levels, protein synthesis and essential metabolic processes (34).

Based on our observations that EGFRvIII expression results in increased autophagy activation in metabolically challenged cells, we hypothesized that the increased survival and growth of EGFRvIII-expressing cells and tumours during stressful conditions is supported by autophagy. Targeting autophagy could therefore be a potential tool to lower tolerance to metabolic stresses and increase cell killing of EGFRvIII-expressing GBM cells.

Materials and Methods

In vivo experiments

Animal facilities and experiments were in accordance with local institutional guidelines and approved by the local animal welfare committee. Experiments were performed as described previously (33). Briefly, tumours were grown in NMRI-nu (nu/nu) female mice (Charles River). Cells in matrigel (BD Biosciences, 354234) were injected subcutaneously (1.5×10^6 cells). For the generation of primary human xenografts, a small piece of biopsy (approx. 1 mm^3) was implanted for the generation of the first generation tumour (growth until 1 cm^3). This tumour was cut into multiple pieces, implanted in recipient mice and experiments were subsequently performed on these 2nd generation tumours. Tumour size was assessed by caliper measurement in 3 orthogonal diameters. Mice treated with CQ (Sigma Aldrich, C6628) received 60 mg/kg CQ for 7 consecutive days. For radiation treatment, tumours were positioned in the irradiation field using a custom-built jig and irradiated with a single dose of 10 Gy (15 MeV e-) using a linear accelerator (Varian, Palo Alto, CA, USA)(35) Number of animals used are listed in the respective figure legend.

Patient studies

The retrospective analyses and the establishment of patient derived xenografts were approved by the respective local medical ethics committees. All patients with a primary GBM, treated in Maastricht with radiotherapy between 2007 and 2012 with sufficient histological tissue available and who had completed the planned treatment with temozolomide ($75 \text{ mg/m}^2/\text{day}$) and radiotherapy (60Gy), were included in the analysis (Fig. 6B and table 1). The effect of CQ on prognosis in EGFRvIII-expressing GBM was evaluated on 43 GBM patients (of which sufficient tumour tissue was still available) that were included in a randomized, placebo controlled phase II trial (Fig. 6C and table 2) (36).

Reagents and cell lines

Unless specified otherwise, all reagents were obtained from Sigma Chemical Co. (the Netherlands) and all electrophoresis reagents were from Bio-Rad (the Netherlands). MEM- α medium was obtained from Invitrogen (10370070). Glutamax-I (35050038) and pyruvate (11360039) were obtained from Life Technologies, DMEM (BE12-604F) and fetal bovine serum (FBS) were from GE-Healthcare. Glucose determination kits were purchased from Biosentec (010). Cell lines were obtained from and maintained as described by ATCC (HTB-14 (U87) and HTB-17 (U373)), negative for mycoplasma contamination (Lonza Mycoalerts, LT07-318). The cells' origin was authenticated by STR analysis (Identicell). Cell lines that endogenously express EGFRvIII (DKMG and BS153) were kindly provided by Malte Kriegs and Nina Struve (Hamburg, Germany) and maintained as described previously (37). For doxycycline (Sigma, D9891) inducible knockdown of *Lc3b*, shRNA [TTTCTCACTCTCATACACTCT] (Sigma Aldrich, TRCN0000152696) was expressed from Tet-pLKO-puro (addgene, 21915; depositor Dmitri Wiederschain (38)) as described previously (39). *ATG7* knockdown was induced using pTRIPZ lentiviral delivery of shATG7 [ATACAGTGTCCAATAGCTGGG] (Dharmacon). Dominant negative ULK1^{K46I} was used as described previously (40). Differences in autophagic flux were confirmed using mCherry-EGFP-LC3 expression from a pBabe-puro vector (addgene, 22418; depositor J. Debnath) (41).

Western blotting

Cells were lysed and processed as described previously (7) and proteins were separated through SDS-PAGE. After transfer, proteins were probed with antibodies against EGFR, EGFRvIII runs at lower height (Santa Cruz Biotechnology, sc-03), MAP1LC3B (Cell Signaling Technology, 2775S), ACTB/actin (MP Biomedicals, 08961001), p-EGFRvIII (Cell Signaling Technology, 3777), p-MAPK/ERK (Cell Signaling, 4370) or tubulin (Cell Signaling Technology, 2146). Bound antibodies were visualized using HRP-linked anti-rabbit (Cell Signaling Technology, 7074S) or anti-mouse (Cell Signaling Technology, 7076S) antibodies.

Immunofluorescence and immunohistochemistry

For immunofluorescence, cells were grown on glass coverslips (VWR, 470019-016). After exposure, cells were washed and fixed in 4% paraformaldehyde (Sigma Aldrich, p6148). After permeabilization, cells were probed with anti-MAP1LC3B (Cell Signaling Technology, 2775S) followed by anti-rabbit Alexa Fluor 488 (Invitrogen, A11006). For EGFRvIII expression determination in tumours, formalin-fixed paraffin-embedded tissue slides were probed with antibodies directed against EGFRvIII (clone L8A4, kindly provided by Dr. Bigner [Duke University Medical Center, Durham, NC]).

For assessment of microenvironmental changes in the xenograft studies, frozen, acetone-fixed sections were stained by using anti-pimonidazole (Hypoxyprobe Inc, HP4-100kit), 9F1 (rat monoclonal antibody to mouse endothelium, Department of Pathology, Radboud University Medical Center) or anti-BrdU (Sigma-Aldrich, B8434; as described previously (33)). For quantitative analysis, the slides were scanned with a computerized digital image processing system by using a high-resolution intensified solid-state camera on a fluorescence microscope (Axioskop; Zeiss, the Netherlands) with a computer-controlled motorized stepping stage (33).

Proliferation assessment

Cells were seeded in a 24-well culture plate (Greiner Bio One, 662160) and incubated under normal, serum starved or hypoxic conditions (0.2% O₂). CQ was used at a concentration of 5 µg/ml (Sigma Aldrich, C6628). Cellular density was determined using Cristal violet staining.

Clonogenic survival assay

Eight h after counting and seeding, cells were exposed to serum-free medium or hypoxia (O₂ < 0.02%, Don Whitley, VA500) as described previously.(42) After exposure the medium was replaced with normal medium and cells were incubated under standard culture conditions until colonies formed (10 days). Colonies were visualized using 0.4% methylene blue in 70% ethanol staining. Only colonies consisting of ≥50 cells were counted.

Statistics

Statistical analyses were performed using graphpad software. When comparing 2 conditions unpaired t-tests were used. For multiple comparisons, one way-ANOVA with Bonferroni corrections were used. p-values <0.05 were considered statistically significant.

Results

EGFRvIII-expressing cells are highly dependent on autophagy during starvation

EGFR is often overexpressed or mutated in diverse cancer types and especially in glioblastoma. One of the most commonly found mutations in EGFR is the constitutive active deletion variant EGFRvIII, which contributes to increased intrinsic radioresistance but also increases tolerance to microenvironmental factors that contribute to therapy resistance (e.g., hypoxia) (24-26,43,44). The tumour microenvironment is characterized by the presence of regions that are low in oxygen (hypoxia) and nutrient supply. Previously we and others have shown that cells deprived of nutrients or oxygen rapidly activate

autophagy (31-33,45). Considering the high proliferation rate and nutritional demand of EGFRvIII-expressing cells, (46) we explored autophagic activity in EGFRvIII-expressing cells. Immunohistochemical analysis during serum-starved conditions revealed more and larger autophagosomes in the EGFRvIII-expressing cells compared to the control cells (Fig. 1A), suggesting changes in autophagic activity.

The gold standard in autophagy activity determination, is the measurement of autophagic flux. In short, during autophagy LC3B is partially degraded. Blocking lysosomal function prevents this degradation and the accumulation of this protein during these conditions reflects autophagic activity (flux). To achieve lysosomal inhibition, cells were exposed to nutrient deprivation in the absence and presence of the lysosomotropic drug, chloroquine (CQ, 5µg/ml). A faster and more pronounced induction of autophagy, as indicated by the accumulation of LC3B-II, was observed in the EGFRvIII-expressing U373 (Fig. 1B) and U-87 MG cells (Fig. 1C). Exposure to afatinib, an irreversible tyrosine kinase inhibitor, decreases autophagic flux in EGFRvIII-expressing cells in a dose-dependent manner, indicating that the increased autophagic flux in EGFRvIII-expressing cells during starvation was EGFRvIII-activity dependent (Fig. S1A).

To determine if the higher autophagic flux contributed to growth advantages of EGFRvIII-expressing cells, cellular proliferation during normal and starvation conditions was determined. CQ addition had no significant effect on proliferation of either control or EGFRvIII-expressing cells during normal culture conditions (Fig. 1D), indicating that the used concentration was non-toxic at this dose. During starvation, EGFRvIII-expressing cells proliferated faster compared to control cells (Fig. 1E, $p < 0.05$). This growth advantage was abrogated by the addition of CQ to the culture medium. To assess whether autophagy inhibition alters survival of EGFRvIII-expressing cells, clonogenic survival after serum deprivation was determined. Serum deprivation did not significantly change clonogenic survival of control or EGFRvIII-expressing cells (Fig. 1F). However, CQ addition resulted in increased cell death of EGFRvIII-expressing cells ($p < 0.05$ Control + CQ versus EGFRvIII + CQ at $t = 4$). Although CQ has been widely used as an inhibitor of autophagy, its effects are not solely restricted to the inhibition of autophagy. To confirm if the observed effects were dependent on autophagy, we engineered cells expressing a doxycycline-inducible shRNA directed against *LC3B*. In agreement with an autophagy dependence, targeting *LC3B* sensitized cells to serum deprivation. In line with our pharmacological data, this effect was most pronounced in EGFRvIII-expressing cells (Fig. 1G, $p < 0.05$).

Taken together, these data indicate that EGFRvIII-expressing cells activate autophagy to a higher extent and are highly dependent on autophagy for survival in response to serum deprivation.

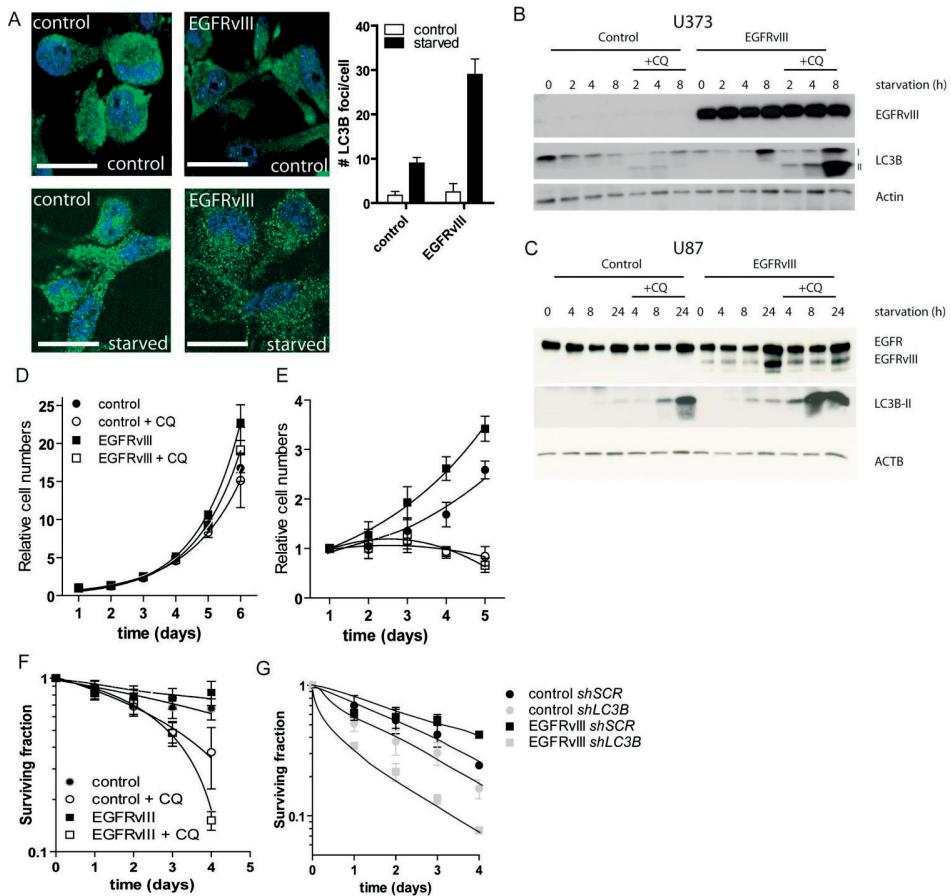


Figure 1. EGFRvIII-expressing cells display increased autophagy activation and dependence during serum-starved conditions.

(A) EGFRvIII-expressing cells display more and larger autophagosomes during starvation as assessed by immunofluorescence (nuclei [DAPI] in blue, LC3B in green). Scale bars: 10 μ m. Immunoblot analysis of control and EGFRvIII-expressing cells reveal elevated autophagic flux during serum-starved conditions in EGFRvIII-expressing U373 (B) and U87-MG (C) cells. Proliferation curves under normal (D) and serum-starved (E) conditions show that CQ (5 μ g/ml) does not influence growth under normal conditions, but can abrogate the growth advantage of EGFRvIII-expressing cells during starvation. $n=3$, mean \pm SEM. Clonogenic survival assessment after serum starvation with or without addition of CQ (F) or expression of a shRNA targeting LC3B (G) indicate that the elevated survival advantage of EGFRvIII-expressing cells is autophagy dependent. $n=3$, mean \pm SEM. SCR, scrambled control.

EGFRvIII-expressing cells depend more on autophagy during hypoxia

To assess if the enhanced autophagic response was restricted to serum deprivation or also applies to other types of metabolic stress present within the tumour microenvironment, we assessed the autophagic response and dependency on autophagy during hypoxic conditions. Similar to serum deprivation, EGFRvIII⁺ cells displayed an enhanced

autophagic flux compared to the control cells as indicated by faster LC3B-II accumulation in the presence of CQ (5 $\mu\text{g/ml}$) in U373 (Fig. 2A) and U-87 MG cells (Fig. 2B). This was further confirmed using tandem mCherry-EGFP-LC3B expression in U373 cells. Although no clear differences could be observed during normal culture conditions (not shown), after 16 h hypoxia exposure, EGFRvIII⁺ cells displayed mostly red foci whereas EGFRvIII⁻ cells also displayed green/yellow foci (Fig. 2C). These data indicate faster processing of autophagosomes in EGFRvIII⁺ cells and support the flux analysis results seen by immunoblot.

To assess the dependency on autophagy for the survival of EGFRvIII-expressing cells, clonogenic survival after hypoxia exposure was determined in the presence of CQ (Fig. 2D) or after expressing a short hairpin that targets *LC3B* (Fig. 2E), or *ATG7* (Fig. 2F) or after overexpression of dominant negative ULK1^{K46I} (Fig. 2G). As reported previously, (12) EGFRvIII-expressing cells displayed a survival advantage in comparison to control cells, yet EGFRvIII-expressing cells were sensitized to hypoxia ($\text{O}_2 < 0.02\%$) more than control cells in the presence of CQ or after targeting autophagy genetically (Fig. 2C and D, both $p < 0.05$ at $t = 3$ and/or -4 ; Fig. 2F and G, $p < 0.05$).

In addition to analysis of cells that ectopically express EGFRvIII, we explored the autophagy dependence of glioblastoma cells that endogenously express EGFRvIII. EGFRvIII-enriched populations were obtained after cell sorting (37). This way BS153 and DKMG isogenic cell populations have been obtained that lack EGFRvIII expression or are highly enriched for EGFRvIII expression (Fig. 2H). Also endogenous EGFRvIII⁺ cells displayed enhanced autophagic flux during hypoxia in BS153 (Fig. 2I) and DKMG (Fig. 2J) isogenic cell lines. This was further confirmed using mCherry-EGFP-LC3B expression in these cells. EGFRvIII⁺ cells displayed mostly mCherry-positive foci whereas EGFRvIII⁻ also displayed yellow/green foci, suggesting a more rapid turnover of autophagosomes in EGFRvIII⁺ BS153 (Fig. 2K) and DKMG (Fig. 2L) cells. Due to their migratory character both BS153 and DKMG cells failed to form colonies. Therefore, the sensitivity of these cells to CQ (5 $\mu\text{g/ml}$) addition during hypoxia was assessed by crystal violet staining after exposure to hypoxia. During ambient air incubation, this concentration of CQ did not influence cell proliferation (Fig. S1B and C). As observed in ectopically expressing EGFRvIII⁺ cells, endogenously expressing EGFRvIII⁺ cells displayed a survival advantage during hypoxia in BS153 (Fig. 2M) and DKMG (Fig. 2N) cells. This survival advantage was fully abrogated in the presence of CQ. These data indicate that cells are highly dependent on autophagy for survival during hypoxia after ectopic, but also endogenous, expression of EGFRvIII.

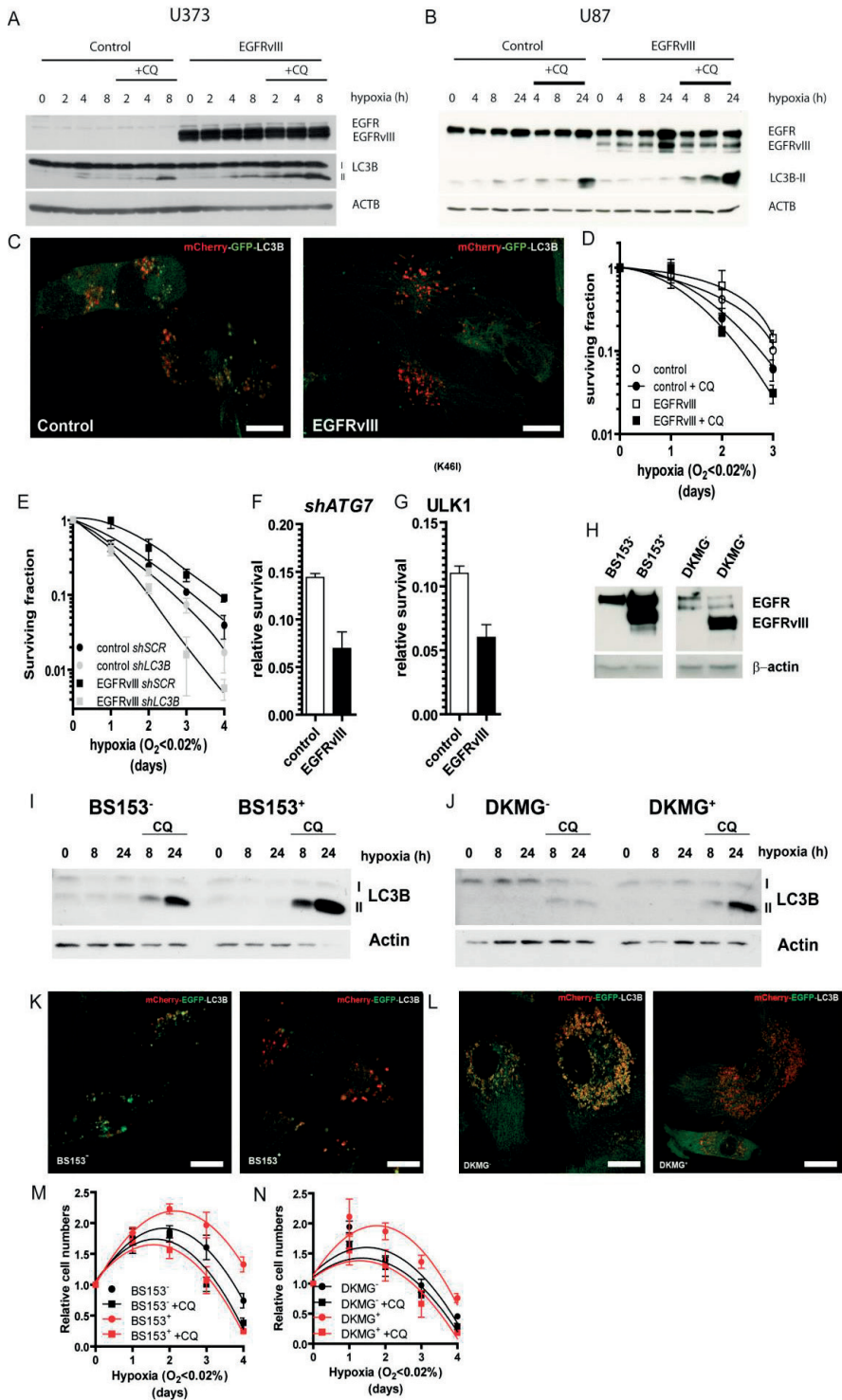


Figure 2. Inhibition of the elevated autophagic response in EGFRvIII-expressing cells abrogates their survival advantage during hypoxia. Immunoblot analysis indicates elevated autophagy activation in EGFRvIII-expressing U373

(A) and U87 (B) cells during hypoxia. (C) Tandem GFP-mCherry-LC3B expression indicates elevated autophagic flux in EGFRvIII⁺ cells during hypoxia (24 h O₂<0.02%). Scale bars: 2 µm. Autophagy inhibition either through CQ addition (5 µg/ml) (D) or shRNA expression directed against *LC3B* (E) abrogates the survival advantage of EGFRvIII-expressing cells as assessed by clonogenic survival assay. n=3, mean ± SEM (F) Targeting *Atg7* through expression of a shRNA (G) or ULK1 through expression of dominant negative ULK1^{K46I} results in loss of survival advantage of EGFRvIII⁺ cells as determined by clonogenic survival assay after 48 h hypoxia (O₂<0.02%). n=3, mean ± SEM (H) EGFR and EGFRvIII expression of BS153 and DKMG cell populations. Autophagic flux determination in (I) BS153 and (J) DKMG cells during hypoxia. Tandem GFP-mCherry-LC3B expression indicates elevated autophagic flux in EGFRvIII⁺ (K) BS153 and (L) DKMG cells during hypoxia (24 h O₂<0.02%). Scale bars: 2 µm. Autophagy inhibition through CQ addition (5µg/ml) abrogates the survival advantage of EGFRvIII⁺ (M) BS153 and (N) DKMG cells as assessed by crystal violet staining after hypoxia exposure. n=3, mean ± SEM.

EGFRvIII-expressing xenografts depend on autophagy for accelerated regrowth after therapy

To investigate the higher dependency of EGFRvIII-expressing cells on autophagy during metabolic stresses *in vivo*, control and EGFRvIII-expressing tumour xenografts were grown in nude mice (Fig. 3A) and treated with CQ (60 mg/kg daily). In line with previous results, (12) EGFRvIII tumours displayed accelerated growth compared to control tumours (Fig. 3A). Interestingly, 7-day CQ treatment resulted in decreased growth (Fig. 3B; 20% increase in doubling time for control tumours and 30% increase for EGFRvIII expressing tumours, both $p<0.05$). EGFRvIII expression was validated by immunohistochemistry (Fig. 3C).

CQ treatment changes the tumour microenvironment by decreasing the hypoxic fraction through reduced hypoxia tolerance (33) and increased perfusion (47). Our *in vitro* data suggest that EGFRvIII-expressing cells were more dependent on autophagy for survival during hypoxia (Fig. 2). In line with the observed increased intrinsic tolerance of EGFRvIII-expressing cells to hypoxia (Fig. 2 and ref. (12)), EGFRvIII-expressing tumours displayed a higher hypoxic fraction compared to control tumours as determined by pimonidazole immunohistochemistry (Fig. 3D). In both control tumours and EGFRvIII-expressing tumours CQ treatment resulted in a decrease in tumour hypoxia (Fig. 3D; $p<0.05$ in control, $p<0.01$ for EGFRvIII tumours), although this effect was most pronounced in EGFRvIII-expressing tumours.

As expected, cellular proliferation, as determined by BrdU incorporation, was higher in EGFRvIII-expressing tumours compared to control tumours (Fig. S2A), but was unaffected by CQ treatment, indicating that hypoxic cells contribute very little to proliferation in the tumour. In control tumours, CQ treatment increased vessel density ($p<0.05$) and may have contributed to the reduction in tumour hypoxia (Fig. S2B). In contrast, EGFRvIII tumours showed a high vessel density when untreated, but there CQ treatment reduced, although to a limited extent (18%), the vessel density ($p<0.05$). These results suggest that the effect of CQ on vessel function and abundance is phenotype depend-

ent. No differences in perfused vessels in either of the tumours were observed (Fig. S2C). In contrast, the necrotic fraction increased significantly after CQ treatment in EGFRvIII-expressing tumours (2.3 fold, $p=0.01$), but not in control tumours (Fig. 3E), suggesting that CQ treatment leads to cell death of EGFRvIII-expressing cells *in vivo*.

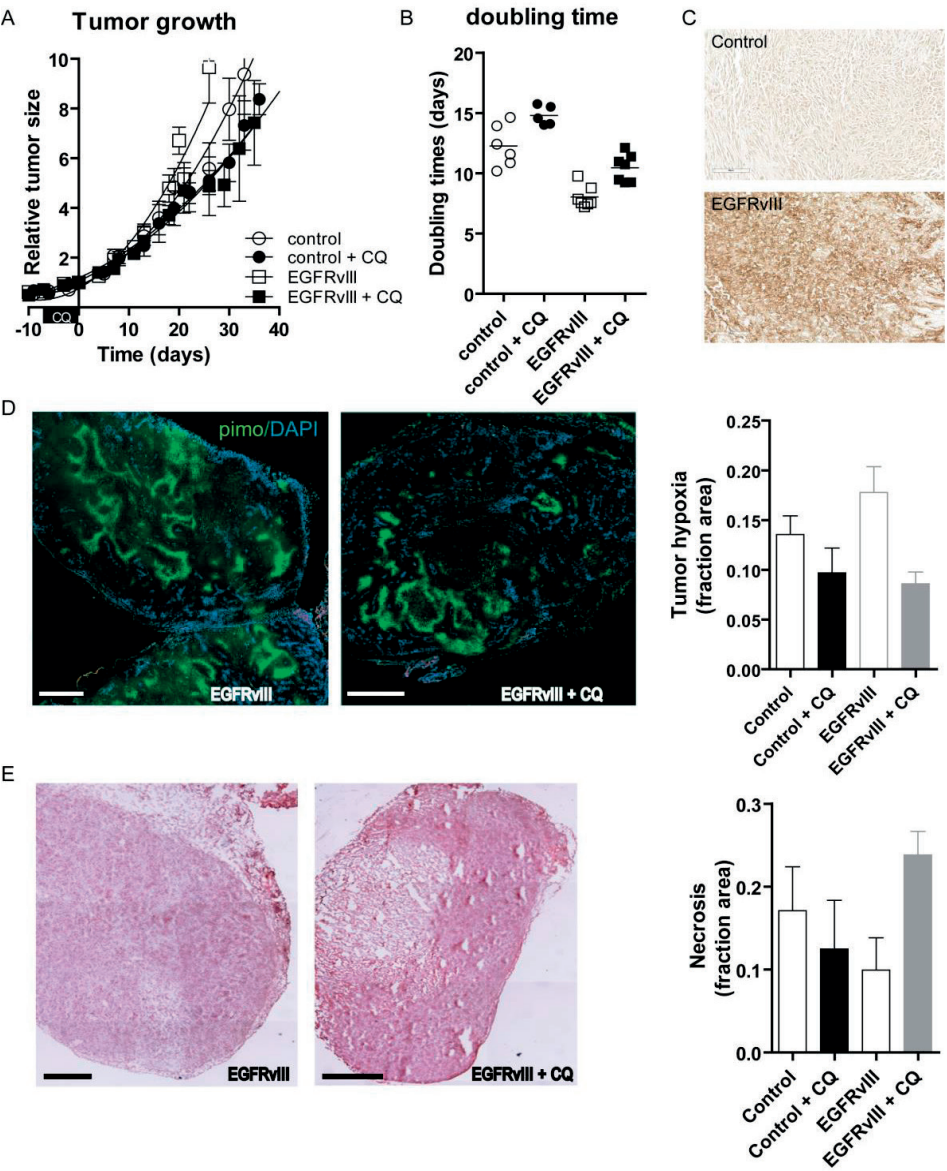


Figure 3. CQ treatment reduces tumour growth and tumour hypoxia and increases tumour necrosis in EGFRvIII-expressing tumours.

(A) Growth curves of control U373 xenografts (open circles, n=6) or treated from day -7 to 0 with CQ (60 mg/kg) (filled circles, n=5) and EGFRvIII-expressing U373 xenografts (open squares, n=7) treated with CQ (filled squares, n=7). (B) Doubling times of the corresponding tumours. (C) Immunohistochemical staining for EGFRvIII on control (upper panel) and EGFRvIII-expressing xenografts (lower panel). Scale bars: 2 mm. (D) (Left panel) Micrographs of pimonidazole-DAPI staining prior to and after 7 days CQ treatment on EGFRvIII-expressing tumours. (Right panel) Quantification of the tumour hypoxic fraction (control n=6, control + CQ n=6, EGFRvIII n=5, EGFRvIII + CQ n=5). (E) (Left panel) Micrographs of hematoxylin and eosin staining prior to and after 7 days CQ treatment on EGFRvIII-expressing tumours. (Right panel) Quantification of tumour necrosis (control n=6, control + CQ n=6, EGFRvIII n=5, EGFRvIII + CQ n=5).

Hypoxic cells contribute to radiotherapy resistance; we therefore determined whether the decrease in hypoxia after CQ administration could sensitize EGFRvIII-expressing tumours to irradiation. Control and EGFRvIII tumour bearing mice were treated with CQ for 7 days prior to irradiation and tumour regrowth was monitored. As expected, growth of control tumours was significantly delayed after a single, tumour specific, dose of 10 Gy and could be further delayed by CQ pre-treatment (Fig. 4A). EGFRvIII-expressing tumours respond faster to irradiation, often an indication of a different type of cell death(48) but also regrow faster. Yet the effect of CQ pre-treatment on EGFRvIII-expressing tumours postponed regrowth even further (Fig. 4A). To illustrate the differences in radiation sensitivity of the individual tumours, the data are replotted in Fig. 4B as Kaplan–Meier plots. Tumour irradiation extended the median survival of mice with control tumours from 20 to 31 days, and CQ pre-treatment increased median survival further to 40 days. The effect of CQ addition in EGFRvIII-expressing tumours was much larger as irradiation extended survival from 15 to 25 days, but in combination with CQ pre-treatment this survival was extended to 48 days (Fig. 4B). This effect was even more pronounced after a larger irradiation dose (15 Gy), showing an additional increase from 61 to 108 days after CQ treatment prior to irradiation (Fig. S3).

Expression of EGFRvIII on GBM cells is rapidly lost when cultured *in vitro*, suggesting a survival and/or selection advantage for EGFRvIII-expressing cells in the tumour micro-environment. For this reason most studies on EGFRvIII are performed in established GBM cell lines in which EGFRvIII is ectopically expressed. These cells and xenografts might behave different than the original tumours as these cells are clearly not dependent on EGFRvIII signalling for survival or growth, and expression levels of EGFRvIII may be much higher than endogenous levels. We have therefore generated primary tumour xenografts directly derived from a cancer patient (patient derived xenograft; PDX), with an EGFRvIII-positive GBM. These primary tumour xenografts are thought to better resemble and reflect the original tumour and its response to therapy. After growth as a xenograft, EGFRvIII expression was retained over generations, as determined by EGFRvIII-specific immunohistochemistry (Fig. 4C) and a substantial hypoxic fraction was observed (Fig. 4D, mean \pm Stdev 0.15 ± 0.04 , n=6). Similar to the ectopically EGFRvIII-expressing tumours, CQ administration reduced tumour growth (18 to 28 days doubling time $p < 0.05$) in endogenously expressing EGFRvIII-positive PDX (Fig. 4E), which resulted in an increase in median survival from 32 to 40 days (Fig. 4F). Tumour irradiation in-

creased median survival to 66 days and could be increased by CQ administration to 102 days post treatment. Together, these data indicate that autophagy is a therapeutic vulnerability of EGFRvIII-expressing GBM that can be exploited by CQ administration.

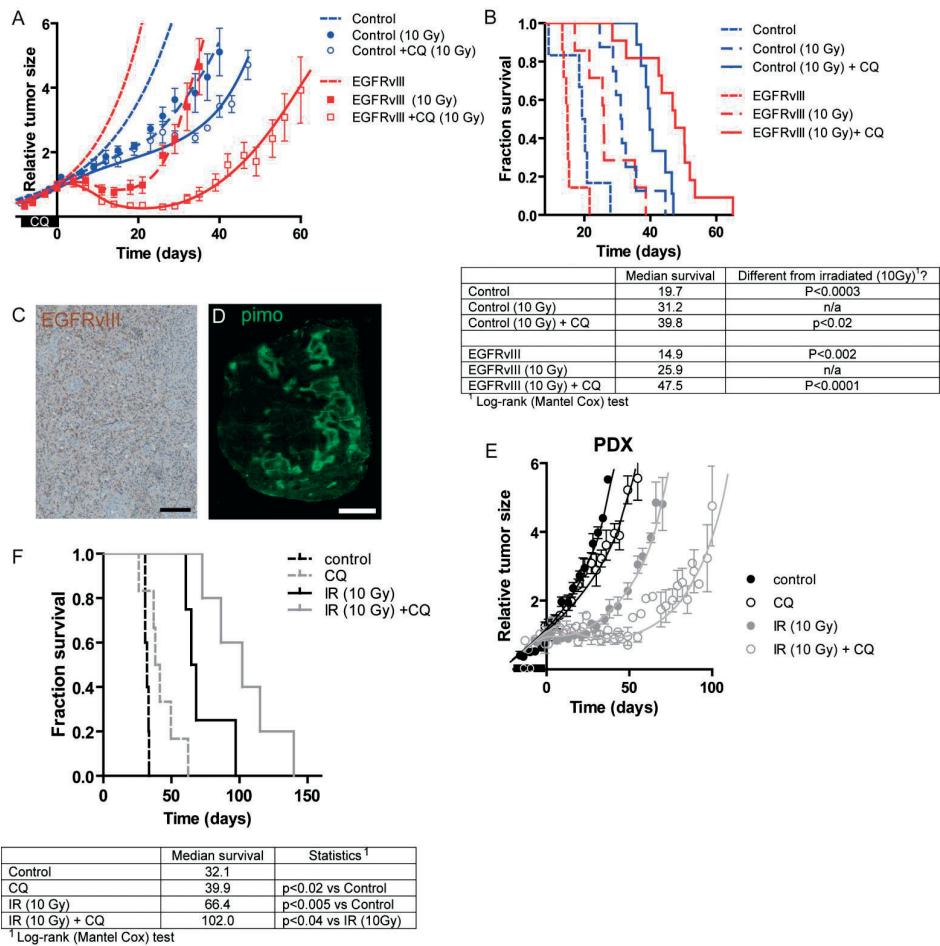


Figure 4. CQ treatment sensitizes EGFRvIII-expressing tumours to irradiation.

(A) Growth curves of U373 xenografts: untreated (dashed lines; control [blue, n=6], EGFRvIII [red, n=7]), treated with a single, tumour-specific dose of 10 Gy at t=0 (interrupted line; control [blue, n=8], EGFRvIII [red, n=7]) or irradiated after 7 days CQ (60 mg/kg) pre-treatment from t = -7 to t = 0 and irradiated with a single, tumour-specific dose of 10 Gy at t = 0 (solid lines; control [blue, n=9], EGFRvIII [red, n=11]). (B) Kaplan-Meier representations of the time to reach 4x the treated volume (from t = 0). Immunohistochemical staining for (C) EGFRvIII (scale bar: 50 µm) and (D) pimonidazole of PDX (scale bar: 2 mm), indicating EGFRvIII positivity and a substantial hypoxic fraction. (E) Growth curves of the PDX untreated (black closed circles, n=5), treated with CQ (60 mg/kg) for 7 days from t = -7 to t = 0 (open circles, n=5), treated with a single, tumour-specific dose, of 10 Gy at t = 0 (gray closed circles, n=4) and treated with CQ (60 mg/kg) for 7 days from t = -7 to t = 0 followed by a single, tumour-specific dose, of 10 Gy at t = 0 (gray open circles, n=5). (F) Kaplan-Meier representations of the time to reach 4x the treated volume (from t = 0).

EGFRvIII-expressing cells are dependent on autophagy due to a high metabolic demand

As EGFRvIII-expressing cells appear to be more dependent on autophagy for their survival during metabolic stress, we hypothesized that autophagy supports the high metabolic demand of EGFRvIII-expressing cells.(46) We therefore assessed glucose abundance in the medium of control and EGFRvIII-expressing cells. Although glucose consumption was higher under normal culture conditions in EGFRvIII-expressing cells, the addition of CQ (5 $\mu\text{g/ml}$) did not change glucose consumption under ambient oxygen conditions (Fig. 5A, $p < 0.05$ at $t = 24$). As expected, glucose consumption increased during hypoxia (Fig. 5A). Interestingly, CQ addition did not change glucose consumption of control cells, but increased glucose consumption by EGFRvIII-expressing cells (Fig. 5A). Comparable to CQ, inhibition of autophagy through targeting *LC3B*, increased glucose consumption in EGFRvIII-expressing cells (Fig. 5B) during hypoxia, but also increased glucose consumption in control cells. This illustrates that besides autophagy, CQ and *LC3B* may also be involved or influence other processes.

The increased glucose metabolism after inhibition of autophagy during hypoxia in EGFRvIII cells, suggests that autophagy is used as an alternative mechanism required in support of the high metabolic demand and energy production. We therefore questioned whether the elevated autophagic flux in EGFRvIII cells during hypoxia is required to meet this demand. We therefore assessed autophagic flux after provision of additive external resources (glucose and pyruvate) for metabolic processing. In control cells, no changes in flux were observed after addition of glucose and/or pyruvate (Fig. 5C). In contrast, in EGFRvIII-expressing cells, addition of pyruvate, but not glucose, rescued the elevated autophagic flux. We hypothesized that failure to meet the high metabolic demand, for example through autophagy inhibition, results in cell death of EGFRvIII-expressing cells. To test this, we exposed control and EGFRvIII-expressing cells to hypoxia for 48 h in the presence or absence of CQ (Fig. 5D) or short hairpins targeting *LC3B* (Fig. 5E), supplemented the culture medium with additional pyruvate and assessed clonogenic survival. Interestingly, the addition of pyruvate rescued cell death in autophagy-deficient EGFRvIII-expressing cells ($p < 0.01$ in Fig. 5D and E), but not in control cells. This illustrates the therapeutic potential of targeting the high metabolic demand of EGFRvIII-expressing cells and tumours through inhibition of autophagy.

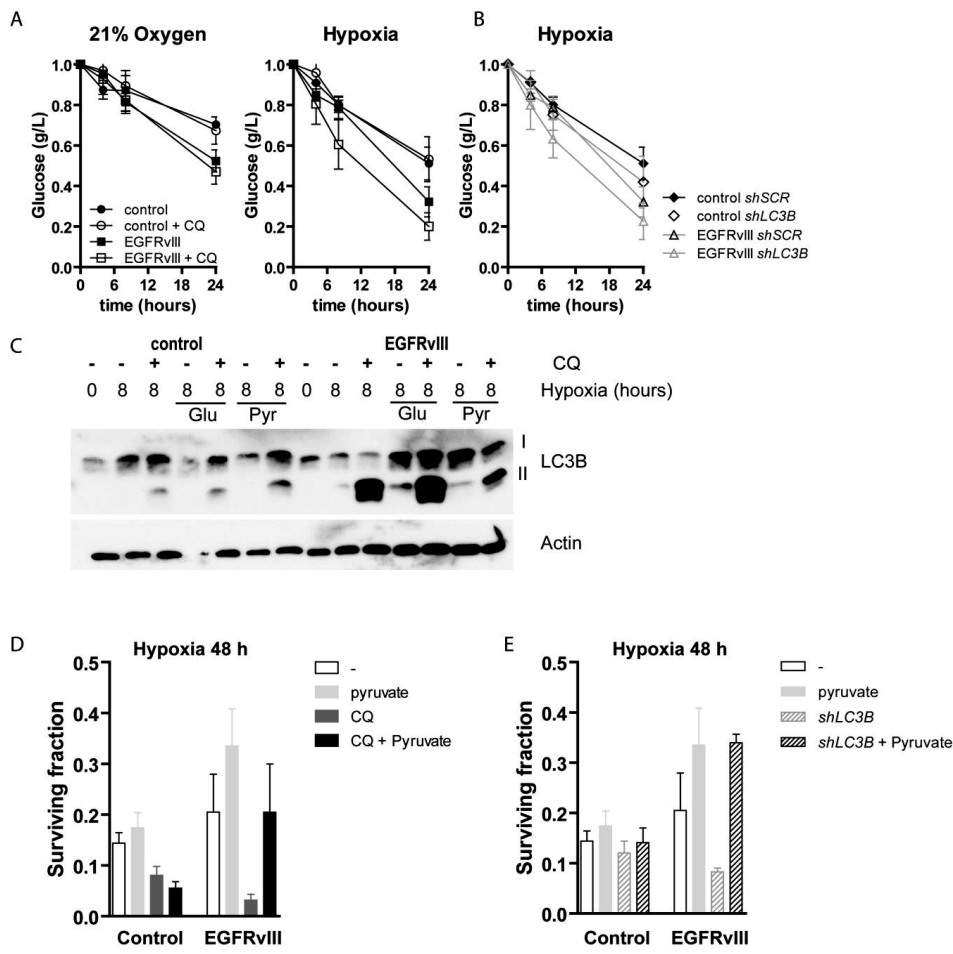


Figure 5. EGFRvIII-expressing cells require autophagy to meet high metabolic demand.

(A) Glucose utilization by control and EGFRvIII-expressing U373 cells under ambient (left) or hypoxic ($O_2 < 0.02\%$) (right) conditions with or without addition of CQ (5 $\mu\text{g}/\text{ml}$). $n=3$, mean \pm SEM. (B) Glucose consumption after expression of a control shRNA or a shRNA targeting *LC3B*. $n=3$, mean \pm SEM (C) Autophagic flux determination in control and EGFRvIII-expressing cells after 8-h hypoxia exposure with additional glucose or methyl pyruvate supplementation (5 mM). (D) Clonogenic survival after 48 h hypoxia ($O_2 < 0.02\%$) exposure in the presence of CQ, methyl pyruvate or CQ and pyruvate. ($O_2 < 0.02\%$) $n=3$, mean \pm SEM. (E) Clonogenic survival after 48-h hypoxia ($O_2 < 0.02\%$) exposure in control and LC3B-depleted cells with pyruvate supplemented $n=3$, mean \pm SEM.

Patients with EGFRvIII-positive GBM benefit from CQ treatment

Several reports have been published on the prognostic role of EGFRvIII in GBM patients. Although most reports indicate worse survival for EGFRvIII⁺ GBM patients, others have indicated a different outcome. This difference may depend on the method of EGFRvIII detection either through PCR or immunohistochemistry or on the heterogeneity of the

patient populations and therapeutic interventions included. We therefore selected 57 patients with a primary GBM, recently treated in our institution. In this cohort, 30 (53%) of the tumours were identified to express EGFRvIII through immunohistochemical analysis (Fig. 6A). All patients were treated with TMZ (75 mg per square meter of body-surface area per day) and radiotherapy (total dose 60 Gy, 2 fractions per day). In this very homogeneously treated patient cohort, immunohistochemical detection of EGFRvIII expression confirmed earlier reports (14,49) which indicate significantly worse prognosis for EGFRvIII-expressing GBM patients (Fig. 6B, $p < 0.05$). Patient characteristics are listed in Table 1.

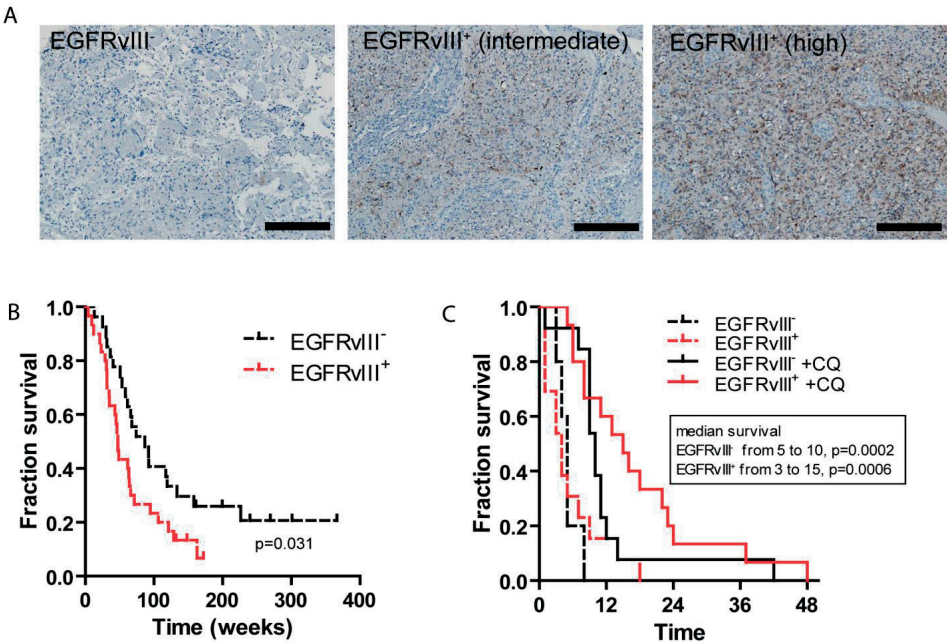


Figure 6. Patients with EGFRvIII-expressing GBM benefit most from concurrent CQ treatment.

(A) Micrographs of immunohistochemically stained biopsy slides of a negative (left) and EGFRvIII-expressing tumours (middle and right). Scale bar: 100 μ m. (B) Kaplan-Meier survival plots of 61 GBM patients after TMZ-radiotherapy treatment, separated based on EGFRvIII expression. (C) Survival plot of 43 GBM patients categorized for EGFRvIII status and CQ treatment. Patients with an EGFRvIII-positive tumour benefit substantially from CQ treatment.

To assess the clinical relevance of our findings in the treatment of EGFRvIII-expressing GBM, we retrospectively analyzed 43 GBM patients that were included in a randomized, placebo-controlled phase II trial (36). Of the analyzed patients, 28 patients received CQ in combination with carmustine and radiotherapy. EGFRvIII expression was assessed immunohistochemically and observed in 22 of 43 (51%) patients. Patient characteristics are listed in Table 2. Although the number of analyzed patients is low, these results are

encouraging as CQ treatment improved median survival from 5 to 10 months in patients with EGFRvIII-negative GBM (Fig. 6C, $p=0.0002$). Strikingly median survival of patients with EGFRvIII-positive GBM increased from 3 to 15 months ($p=0.0006$), confirming our pre-clinical findings and the therapeutic potential of CQ in the treatment of EGFRvIII-expressing tumours.

Table 1. Characteristics of GBM patients treated with TMZ and radiotherapy (60 Gy)

EGFRvIII status	EGFRvIII ⁻	EGFRvIII ⁺
	27 (47%)	30 (53%)
Male	15/27	19/30
Female	12/27	11/30
Karnofsky performance (pre-surgery)		
60 - 70	0 (0%)	2 (7%)
80 – 90	8 (30%)	19 (63%)
100	19 (70%)	9 (30%)
TMZ (75 mg/m ² /day)	27/27 (100%)	30/30 (100%)
Radiotherapy (60 Gy)	27/27 (100%)	30/30 (100%)

Table 2. Characteristics of GBM patients

EGFRvIII status	no CQ treatment		CQ treatment	
	vIII ⁻	vIII ⁺	vIII ⁻	vIII ⁺
Male	5/8	3/7	10/13 (77%)	6/15 (40%)
Female	3/8	4/7	3/13 (23%)	9/15 (60%)
Karnofsky performance (pre-surgery)				
< 70	0 (0%)	1 (14%)	0 (0%)	1 (7%)
70 – 80	7 (87%)	6 (86%)	7 (54%)	7 (47%)
90 -100	1 (13%)	0 (0%)	6 (46%)	7 (47%)
Karnofsky performance (post-surgery)				
< 70	1 (13%)	2 (29%)	0 (0%)	0 (0%)
70 – 80	1 (13%)	1 (14%)	1 (8%)	3 (20%)
90 -100	6 (74%)	4 (57%)	12 (92%)	12 (80%)
2nd surgery	4/8 (50%)	2/7 (29%)	2/13 (15%)	2/15 (13%)
Radiotherapy	3/8 (38%)	2/7 (29%)	8/13 (62%)	6/15 (40%)

Discussion

Our data confirmed that the expression of EGFRvIII indicates a poor prognosis for GBM patients due to increased therapy resistance, increased proliferation and accelerated tumour growth. Furthermore, EGFRvIII-expressing cells are more resistant to metabolic stresses and result in an increased hypoxic fraction of the tumour. As tumour hypoxia is a major limiting factor in successful cancer treatment,(27) targeting hypoxia in EGFRvIII-

expressing tumours may therefore be essential in acquiring improved tumour control and ultimately tumour cure. Here we reveal that expression of EGFRvIII leads to increased autophagic flux during metabolic stresses such as hypoxia and starvation. In line with previous reports, EGFRvIII-expressing cells proliferate faster and display increased survival during these stresses (10-13). We show that these advantages can be abrogated through inhibition of autophagy either through genetic or pharmacological targeting. *In vivo*, EGFRvIII tumours display increased tumour growth and faster tumour regrowth after radiotherapy, effects that are likely to contribute to the poor outcome of EGFRvIII⁺ GBM patients. Importantly, CQ treatment inhibits the accelerated growth and regrowth of EGFRvIII tumours. As observed previously, (33) CQ treatment results in reduced tumour hypoxia in tumour xenografts, yet we observed that the largest reduction occurs in EGFRvIII-expressing tumours. Strikingly, CQ treatment results in a drastic increase in necrosis of EGFRvIII tumours, but not in control tumours. In addition to lowering the intrinsic tolerance to hypoxia, CQ may induce vessel normalization and increase perfusion, resulting in decreased tumour hypoxia (47). In our experiments, vessel density increased in control tumours but not in EGFRvIII-expressing tumours (Fig. S2). Our data indicates that EGFRvIII expression changes the tumour cell dependence on autophagy to tolerate low oxygen levels. In the absence of autophagy, these cells will die leading to increased tumour necrosis. These results are in line with the enhanced cell death after autophagy inhibition in EGFRvIII-expressing cells, *in vitro* (Fig. 2C and D).

During autophagy, recycled products are transported across the autolysosomal membrane into the cytoplasm. These metabolites can be reused in protein and ATP production (34). In our studies we observed that EGFRvIII⁺ tumours cells displayed increased activation and dependence on autophagy during hypoxia, effects that can be rescued by adding pyruvate to the culture medium. As EGFRvIII⁺ cells consume more glucose (Fig. 5), autophagy may be essential in supporting metabolic processes in EGFRvIII⁺ cells. EGFRvIII may induce the increased glucose uptake and consumption through enhanced RAS signalling. Similar to our observations, oncogenic RAS promotes glucose uptake and utilization in glycolysis (reviewed in ref. (50)). Interestingly, the effect of autophagy inhibition in EGFRvIII⁺ cells on survival and glucose consumption during hypoxia could not be rescued by glucose addition, but was rescued after pyruvate supplementation. This suggests that a) the glycolytic pathway is used to its fullest extent, and b) the high metabolic activity is not used to support ATP production, as pyruvate cannot further be degraded in the citric acid cycle due to the absence of oxygen. In addition to being converted to ATP, pyruvate is able to prevent toxic effects of oxygen radicals and peroxides and indirectly prevents the depletion of glutathione during hypoxia, (51) and may therefore decrease the toxic effects after reoxygenation.

From a clinical perspective, the most important finding of this research is the relatively large effect of CQ on tumours that express EGFRvIII compared to the non-EGFRvIII-expressing tumours. In cell line-derived xenografts, we observed transition of the hypoxic into necrotic areas and a high degree of sensitization to radiotherapy. These

results were further confirmed in patient-derived xenografts. Furthermore, retrospective analysis of EGFRvIII expression in GBM patients treated with carmustine, radiotherapy and surgery revealed a larger improvement in survival for patients with EGFRvIII-positive GBM after concurrent CQ treatment compared to patients with EGFRvIII-negative tumours. Although these initial clinical results are promising, larger cohorts of patients are required to fully understand the therapeutic potential of CQ administration in the treatment of GBM. Recently a phase I/II trial in newly diagnosed GBM has been reported where standard of care (temozolomide and radiotherapy) was combined with concurrent hydroxychloroquine (a derivative of CQ) (52). Due to unfavourable toxicity profiles and dose-limiting toxicity before consistent inhibition of autophagy was observed, no changes, compared to historical data (EORTC trial), in overall survival were observed. Although hydroxychloroquine and chloroquine belong to the same class of 4-aminoquinolines, their effective doses and toxicity profiles differ. Clinical trials to evaluate toxicity and effectiveness (randomized phase II trial) of CQ in combination with temozolomide and radiotherapy are ongoing in our institute (clinicaltrials.gov identifier NCT02378532 and NCT02432417). Based on the findings described in this manuscript, in the phase II trial patients will be stratified based on expression of EGFRvIII.

Interestingly, other mutation types in the RAS-RAF-MAPK/ERK pathway have also been associated with increased dependence on autophagy for survival. For example, HRAS^{G12V} or KRAS^{G12V} transformed cells upregulate their basal autophagy and depend on elevated autophagy for survival during starvation (53). Also the BRAF^{V600E} mutation, which occurs in over 50% of childhood central nervous system tumours, results in elevated autophagy and increased sensitivity to pharmacological or genetic autophagy inhibition in terms of cell viability.(54) Remarkable clinical responses have been observed.

These tumours are often also effectively treated using kinase inhibitors, such as vemurafenib. Unfortunately, development of resistance is often observed. Levy et al. reported that autophagy contributes to vemurafenib resistance. Hence, targeting autophagy can overcome this resistance, and patients with resistant tumours display favourable clinical responses when chloroquine is combined with vemurafenib (55). Combined with our data these results suggest that constitutive signalling through the RAS-RAF-MAPK/ERK pathway creates autophagy dependence in tumour cells. This important information could be used for individualized treatment selection for patients with brain tumours.

In conclusion, our results demonstrate that elevated autophagy activity contributes to the enhanced tolerance to metabolic stresses of EGFRvIII-expressing cells. Targeting this survival mechanism abrogates this advantage and results in enhanced tumour cell killing. Ultimately this downregulation of autophagy activity leads to reduced tumour growth, reduced tumour hypoxia, increased tumour necrosis and sensitization of tumours to radiotherapy. In line with these pre-clinical findings, we observed that patients with EGFRvIII-expressing GBM benefit most from concurrent CQ treatment. Concurrent

EGFRvIII expression triggers a metabolic dependency and therapeutic vulnerability

CQ treatment in combination with conventional therapy seems therefore a promising strategy to improve the outcome for EGFRvIII⁺ GBM patients.

Acknowledgements

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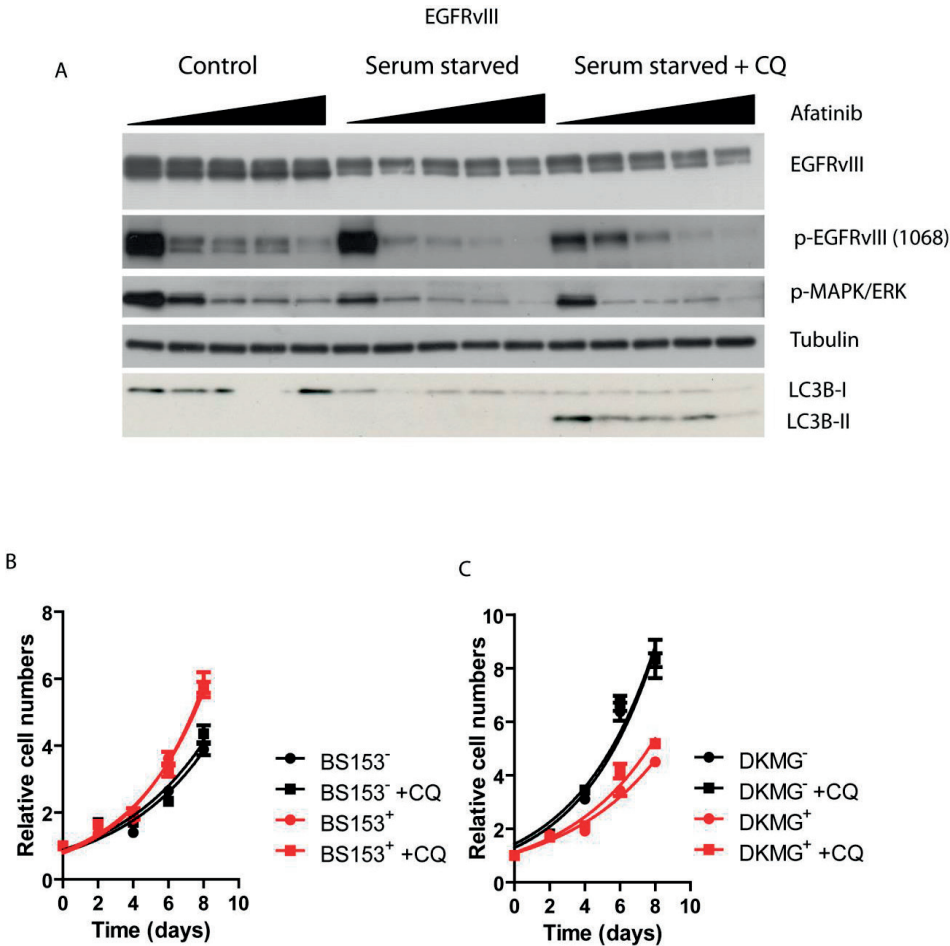
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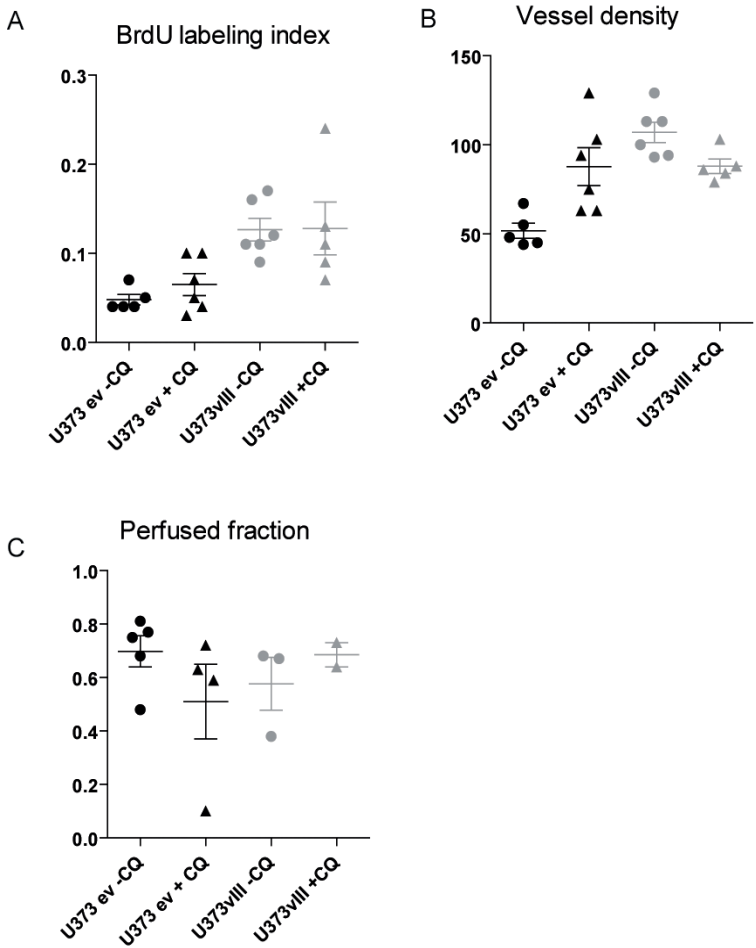
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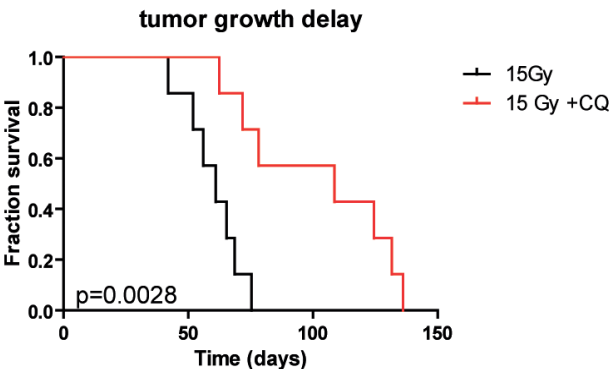
Supplementary figures



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3

Chapter 5

ATG12 expression predicts tumour hypoxia and tumour control in HNSCC

¹Tom G. Keulers, ²Alexander Koch, ¹Hanneke Peeters, ¹Marieke Van Gisbergen, ¹Kim G.M. Savelkouls, ³Nicole Bitsch, ⁴Roel G. Wanders, ⁵Dennis Roop, ⁶Johan Bussink, ²Veerle Melotte, ¹Kasper M.A. Rouschop

¹Department of Radiotherapy, GROW - School for Oncology and Developmental Biology, Maastricht University Medical Centre+, Maastricht, The Netherlands

²Department of Pathology, GROW - School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands

³Cardiovascular Research Institute Maastricht, Universiteitssingel 50, 6229 ER, Maastricht, The Netherlands.

⁴MAASTRO Clinic, Maastricht, The Netherlands.

⁵Charles C. Gates Center for Regenerative Medicine, University of Colorado Denver, Aurora, CO, USA.

⁶Department of Radiation Oncology, Radboud University Medical Center, Nijmegen, The Netherlands.

Chapter 6

Autophagy-dependent secretion: contribution to tumour progression

Tom G.H. Keulers^{1*}, Marco B.E. Schaaf^{2*}, Kasper M.A. Rouschop¹

¹ Maastricht Radiation Oncology (MaastRO) lab, GROW – School for Oncology and Developmental Biology, Maastricht University, Maastricht, the Netherlands

² Cell Death Research and Therapy (CDRT) Laboratory, Department Cellular and Molecular Medicine, KU Leuven, University of Leuven, Leuven, Belgium

*authors contributed equally

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Abstract

Autophagy is best known as a lysosomal degradation and recycling pathway to maintain cellular homeostasis. During autophagy, cytoplasmic content is recognized and packed in autophagic vacuoles, or autophagosomes, and targeted for degradation. However, during the last years, it has become evident that the role of autophagy is not restricted to degradation alone but also mediates unconventional forms of secretion. Furthermore, cells with defects in autophagy apparently are able to reroute their cargo, like mitochondria, to the extracellular environment; effects that contribute to an array of pathologies. In this review we discuss the current knowledge of the physiological roles of autophagy-dependent secretion, i.e. the effect on inflammation and insulin/ hormone secretion. Finally, we focus on the effects of autophagy-dependent secretion on the tumour microenvironment and tumour progression. The autophagy mediated secreted factors may stimulate cellular proliferation via auto- and paracrine signaling. The autophagy mediated release of immune modulating proteins change the immunosuppressive tumour microenvironment and may promote an invasive phenotype. These effects may be either direct or indirect through facilitating formation of the mobilized vesicle, aid in anterograde trafficking or alterations in homeostasis and/or autonomous cell signaling.

Autophagy

Autophagy is a catabolic process required to maintain cellular homeostasis by lysosomal degradation of aged/damaged organelles (e.g. mitochondria), protein aggregates and pathogens. Autophagy commences with the formation of an initial cup-shaped membrane (phagophore) that elongates while (non-) selectively capturing cytoplasmic constituents into a double-membrane vesicle (autophagosome). Ultimately, the autophagosome fuses with a hydrolase and protease containing lysosome for degradation of the content. The end-products are recycled into the cytosol and are reused in processes including protein synthesis and ATP production.

During the last decade, extensive research revealed that at least 38 Autophagy related proteins (ATGs) comprise the core autophagy machinery that mediate initiation, elongation, cargo recruitment and fusion with lysosomes (1). Furthermore, the yeast atg8 orthologues of the LC3/GABARAP protein family fulfil specialized roles in the execution of autophagy (2). This family consists of 7 active members (LC3A (two splicing variants; LC3A-a and LC3A-b), LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2) which share a high mutual homology, including a conserved C-terminal glycine residue for phosphatidylethanolamine (PE) conjugation to allow membrane association. Conjugation of the LC3/GABARAP protein family members to PE is required for expansion and closure of the phagophore. This process is controlled by 2 ubiquitin like conjugation systems, a process closely orchestrated the ATG4, -7, and -3 and the ATG12-ATG5-ATG16L1 complex (9).

Despite the high grade of homology, the protein family members harbour individual roles in autophagy but are associated with autophagy unrelated functions as well like receptor trafficking and (2).

Autophagy (and related defects) is associated with several pathologies, including neuropathologies, Crohns disease and cancer. In cancer, autophagy is considered a double edged sword; i.e. in healthy tissue, autophagy plays a cytoprotective role by maintaining homeostasis through degradation of cytotoxic constituents, which otherwise may trigger tumorigenic events. Nevertheless, once a tumour is formed, autophagy contributes to survival of cancer cells in areas deprived of nutrients of oxygen (hypoxia)(3,4), a common feature of solid tumours that contributes to tumour progression, therapy resistance and metastases formation(5).

Yet, accumulating research shows that the homeostatic role of autophagy and its related proteins is more elaborate than the degradation of cytoplasmic content alone. Autophagy not only contributes to intracellular homeostasis, but also seems to contribute to tissue homeostasis by mediating intercellular communication. Peptides, proteins and hormones that fail to enter the conventional secretory system due to the lack of a leader/secretion signal sequence can be secreted in an autophagy-dependent manner.

In this review we list the current knowledge on the role of the autophagy machinery in autophagy-dependent secretion and specifically focus on factors that may influence tumour progression.

(Un) conventional protein secretion

In eukaryotes, a classical route for proteins to be secreted is the endoplasmic reticulum (ER)-to-Golgi route. Herein, proteins are directed towards the ER by their amino terminal signal peptide (leader peptide) and progress through vesicular flow to the Golgi. In brief, after ER translocation, proteins are oligomerized and packed into carrier vesicles that exit the ER at specialized regions. These vesicles assemble into vesiculo-tubular structure intermediates known as the ER-to-Golgi intermediate compartments (ERGIC) that, by lateral communication sorts proteins for further anterograde flow to the Golgi complex. In the Golgi, proteins are glycosylated to ensure proper protein structure, increase stability and to allow interactions with target proteins (6). In the trans-Golgi network, secretory proteins are sorted into secretory vesicles that deliver their content to the plasma membrane to result in secretion (7). An increasing number of secreted proteins that lack the leader peptide have been identified. These proteins require alternative pathways to be secreted in a regulated fashion. This implies differences in vesicle formation, sorting and transportation. Multiple alternate, non-classical pathways exist and are commonly referred to as unconventional protein secretion and include both non-vesicular and vesicular mechanisms (8). For example, cytosol-residing proteins, including ABC transporter mediated yeast α -factor (9) or mammalian fibroblast growth factor 2 (FGF-2) are directly transported across the plasma membrane. For FGF2 secretion this is presumed to be mediated through PI(4,5)P₂-induced oligomerization followed by membrane insertion and translocation (10). Other proteins are sorted into vesicular intermediates that fuse with the plasma membrane to release their content into the extracellular space ((interleukin 1 β (IL-1 β) and IL-18 (11)), in part dependent on proteins required for autophagy execution (12,13). This suggests that autophagy-proteins are required to produce secretory vesicles or that autophagosomes gain exocytic features. These processes in autophagy-mediated secretion are distinct from its degradative compartment (as reviewed in more detail in (14)). In this context, the trafficking, tethering, docking and plasma membrane fusion events would differ from canonical autophagy and resemble exocytic mechanisms. Herein, secretory vesicles are diverted from the endocytic system to be released from multivesicular bodies (MVB). These secretory vesicles are then transported to the plasma membrane for content release. For example, the interaction between autophagosomes and the endocytic/MVB pathway (reviewed in (15)) are required for acetyl-CoA-binding protein(Acb1)-secretion and requires the fusion of Acb1-containing autophagosomes with endosomes or MVBs before plasma membrane fusion (16).

The involvement of autophagy-related proteins, in protein secretion indicates that cells utilize these effectors in a surprising mechanism of unconventional secretion. Here, we will discuss the data that support a relation between autophagy and autophagy-related proteins with secretory pathways (table 1). First, we will elaborate on the physiological roles of autophagy in secretory events. Second, cancer cells utilize secretory mechanisms to communicate with stroma and surrounding cancer cells, thereby inducing alterations that influence tumour progression including immunoevasion, immunosuppression, auto- and paracrine signalling and angiogenesis.

Table 1: Effects on the tumour microenvironment

Protein(s)	mechanism	Effect	ref
<i>I. Influencing immunogenic cell death to evade immune surveillance</i>			
ATP	ATG5 knockdown	After radiotherapy and MTX exposure “eat me signal” for immune cells. stimulus for DC recruitment, IFN γ -producing CD4 and CD8 T cells	(65)(63) (64)
HMGB1	ATG5 ^{fl/fl}	Promote processing and presentation of tumour antigens by DCs, enhanced immuno surveillance.	(11)
<i>II. Cytokine release and influence on the tumour microenvironment</i>			
IL1-B, IL-6, IL2	GABARAP Knockout mice	Increased secretion by macrophages	(81)
IFN γ	GABARAP Knockout mice	Increased secretion by lymphocytes	(81)
CXCL9, CXCL10 and CXCL11	FIP200 conditional knockout	Enhanced secretion, leading to improved immuno surveillance	(49)
<i>III.a. Pro-metastatic: Driving an invasive behavior of cancer cells</i>			
LIF, FAMC3, DKK3, IL-8	ATG7 knockdown	These factor promote metastasis via MMP2 upregulation (IL-8), epithelial to mesenchymal transition (FAM3C, DKK3, LIF) and promotion of angiogenesis (IL-8 and DKK3)	(83)
	ATG7, ATG12, ATG3 knockdown chloroquine or bafilomycin A1	Autophagy deficient HRAS ^{V12} transformed breast cancer cell lines display reduced invasive protrusions. Conditioned medium of autophagy-proficient cells rescued the invasive phenotype	(82)
IL6, CCL-2, CCL-20, VEGFA, MMP2	3-MA, ATG5 and ATG7 knockdown	TLR3 and TLR4 activation leads to autophagy –dependent secretion of these factors associated with a migratory and invasive phenotype of lung cancer cells.	(85)
IL6	ATG7 or beclin knockdown	Autophagy deficiency lead to an in- or decrease in low or high autophagic breast cancer cells respectively. Autophagy-dependent secretion of IL-6 are able to promote mammosphere formation and may be important in CSC maintenance	(87) (86)
<i>III.b. Pro-metastatic: Proper Weibel-Palade body formation in vascular endothelium to facilitate metastasis</i>			
WPB proteins		Autophagy is important to sustain secretion of WPBs containing proteins that influence tumour progression	(88)
<i>IV. Chemoresistance</i>			
HMGB1		HMGB1 causes doxorubicin resistance in neighboring breast cancer cells.	(91)

Physiological roles of autophagy mediated secretion

The first evidence for the involvement of autophagy-related proteins in protein secretion was obtained in yeast. Acyl coenzyme A-binding protein (Acbp) is secreted through an unconventional secretory pathway that depends on components of the autophagic process (Atg5, Atg7, Atg8, Atg12) (16). Importantly, this study also demonstrated that autophagy connects with compartments (multivesicular bodies) of endosomal trafficking and SNARE-dependent membrane fusion events are both required for the Acbp secretion. Since the discovery of autophagy-dependent secretion in yeast, also several mammalian factors have been identified to be dependent on autophagy-related proteins for their secretion.

Phosphoinositides are membrane lipids with specific recognition domains for the recruitment of cytosolic proteins and are involved in the regulation of membrane transport. So far, seven phosphoinositides have been described. They can be converted into each other by phosphoinositide kinases and phosphatases (17). PIKfyve is one of those converting kinases and has been implicated in membrane transport events. The substrate of PIKfyve, PI(3)P, plays a critical role in the initiation of autophagy and autophagosome maturation (18). Inhibition or depletion of PIKfyve by apilimod or siRNA resulted in decreased autophagic flux, probably caused by an impaired autophagosome/lysosome fusion. Interestingly, prostate cancer cells incubated with the PIKfyve inhibitor apilimod, secrete more autophagy associated proteins (NBR1, P62, OPTN and CACO2 and membrane-bound LC3B), however proteins involved in earlier stages of autophagy were not observed (ATG2A, ATG5, ATG16L). Analysis of density gradients reveal that these autophagy associated proteins are expressed on vesicles and appear as autophagosome subpopulations, suggesting induced secretory autophagy (19).

1. Cytokines and inflammation

Autophagy is able to suppress inflammasome activation through maintaining mitochondrial homeostasis (20). In line, Zhang et al observed increased inflammasome activation in macrophages after sepsis in Gamma (γ)-aminobutyric acid type A receptor (GABAAR)-associated protein (GABARAP) knockout mice (20). Secretion of cytokines is central in inflammasome activity. Well-documented cytokines that are associated with unconventional secretion in an autophagy-dependent manner are interleukin-1 beta (IL-1B) and IL-18. Dupont et al demonstrated in bone-marrow derived macrophages during conditions of induced autophagy (starvation or Torkinib treatment) that secretion of IL-1B increased, which was reduced in the absence of ATG5. Consistent with data obtained from yeast Acbp, similar additional factors were required (as mammalian equivalents of yeast Golgi-associated protein (GRASP55) and small GTPase Rab8a), indicating a conserved system or cooperation (11).

Interestingly, three dimensional STochastic Optical Reconstruction Microscopy (STORM) demonstrated that IL-1B was not only present in the inner vesicle of the autophagosome, but was already recruited to the emerging phagophore. In this, IL-1B is actively translocated across the membrane and accumulated in the intermembrane space of the mature autophagosome (21). This, in contrast to the sequestration of degradable autophagosomal content which is located in the inner vesicle as a result of cytoplasmic engulfment and closure of the phagophore, this novel mechanism indicates differences in cargo recruitment and indicates formation of different vesicles by the autophagy-related proteins that may guide the distinct fate of the produced vesicle.

II. Role of autophagy-dependent secretion in establishing a barrier for infection

Paneth cells are specialized epithelial cells present at the base of the crypts of Lieberkuhn. These cells control the intestinal microbiota through secretion of intracellular granules that contain antimicrobial peptides and lysozyme. Autophagy-proteins are essential for Paneth cell function. Knockdown of crucial autophagy genes (ATG16L1, ATG5 or ATG7 (22,23)) results in alterations in Paneth cell granules, as illustrated by fewer and aberrant granules with diffuse lysozyme patterns and decreased presence of lysozyme in mucus. Due to its relevance in establishing a microbial barrier, the ATG16L1 gene was annotated as a Crohns disease (CD) risk allele (24) and the phenotype is similar to the abnormalities found in Paneth cells of CD patients.

In contrast to CD where the ileum and colon are often affected, ulcerative colitis is restricted to the colon only. Conditional knockdown of ATG7 in the colon (GlcNAc6ST-2-Cre) increased bacterial colonization in crypts. This phenotype was associated with a decreased release of mucin from goblet cells and reduced expression of antimicrobial and antiparasitic peptides (angiogenin 4, interlectin 1 and relm β). Hence, colonic ATG7 knockdown results in increased susceptibility to the development of UC-like colitis (25).

In line with autophagy-dependent secretion by intestinal tissue, ATG5 and ATG16L1 deficiency results in reduced secretion of mucins (MUC5AC) by human tracheal epithelial cells (26).

Together, autophagy-dependent secretion is required to maintain effective anti-bacterial barriers by epithelial cells. In line, ATG16L1 conditional knockout mice (Villin-Cre) are more susceptible to *Salmonella typhimurium* infection (27). Although, beyond the contribution of establishing a barrier function, autophagy is required for limiting bacterial proliferation through ATG16L1 and LC3C dependent degradation of cytosol-residing *Salmonella* (xenophagy) (28). Evidently, autophagy is a prerequisite for different cell types to maintain their function (e.g. mucus secretion) thereby and protection against inflammatory disorders.

III. Insulin secretion

Pancreatic β -cells are specialized in secretion of insulin in response to high levels of blood glucose concentrations. Release of insulin is mediated by the fusion of insulin-containing vesicles with the plasma membrane. These insulin-containing vesicles are transported towards the plasma membrane. Gamma (γ)-aminobutyric acid type A receptor (GABAAR)-associated protein (GABARAP), one of seven mammalian variants of yeast Atg8, which is already known to be implicated in multiple cellular functions including fusion events in autophagy (29) and receptor trafficking (30) has recently been demonstrated to mediate insulin secretion. Herein, GABARAP binds insulin-containing vesicles in a PE conjugation-dependent mechanism (31)). Together with its microtubules-binding feature (residue 10-22 (30)), GABARAP allows vesicle presentation to the motor protein KIF5B (located at microtubules) and facilitates vesicle mobility and insulin release (32).

IV. NPY secretion

In rat adrenal medullar pheochromocytoma derived cells, the neurites display ATG16L at dense-core vesicles. Although these vesicles do not co-localize with LC3, release of peptide hormone neuropeptide Y (NPY) is dependent on ATG16L1 functioning as a Rab33a (which regulates membrane trafficking events) effector. Interestingly, NPY secretion was not altered in cells that were deficient in autophagy by overexpressing ATG4B^{C74A} or knockdown of ATG13 or ULK1 and indicates a highly specialized role for ATG16L1 in hormone secretion that is independent of other canonical autophagy associated proteins (33).

V. Autophagy-mediated secretion of Weibel-Palade bodies in vascular endothelial cells

Vascular injury stimulates endothelial cells to secrete factors to promote repair. Endothelial secretory granules (Weibel-Palade bodies (WPBs)) contain active molecules including von Willebrand factor (vWF), P-selectin, interleukin-8, angiopoietin-2 and endothelin-1. The intracellular WPBs are characterized by striations parallel to its longitudinal axis and are delineated by a membrane. When secreted, the multimeric hemostatic vWF is tethered to the connective tissue to mediate platelet adhesion at sites of vascular injury. The processing of vWF to a mature form is important in the WPB formation. Treatment with chloroquine or bafilomycin A1, that both raise lysosomal pH, and knockdown of ATG7 or ATG5 reduce the number of endothelial WPBs due to incorrect processing of vWF. In line, endothelial specific deletion of *Atg7* in mice led to reduced epinephrine-induced plasma vWF levels and increased bleeding time. Thus, autophagy in endothelial cells aids in hemostasis by proper maturation of WPBs (34).

VI. Osteoclastic bone resorption

Bone remodeling is a lifelong process of bone degradation and formation important for bone healing after injury, bone restructuring and sustaining bone homeostasis. Osteoclasts cooperate with osteoblasts in bone remodeling in which osteoclasts are responsible for bone degradation and reabsorption of mineralized bone matrix. The osteoclasts are large multinucleated cells that are characterized by their ruffled border by which contact area with the bone is increased. Fusion of secretory lysosomes with the ruffled border causes release of matrix-degrading factors including cathepsin K which aids bone matrix degradation. ATG5 or ATG7 deficient osteoclasts lack a normal ruffled border, impaired localization of secretory factors including cathepsin K and eventually have impaired bone resorption. Importantly, development of osteoclasts was not aberrant indicating that the autophagy deficiency led to functional impairment independent of sustaining cellular homeostasis. Moreover, secretory lysosome formation was unaffected, whereas Rab7 (key factor for lysosome fusion events) localization to ruffled border was ATG5 dependent. Together, these data indicated that autophagy-related proteins aid in secretory events at the osteoclast ruffled border by directing fusion of the secretory lysosome with the plasma membrane (35).

In conclusion, autophagy is important for secretory functions of various cell types. Important to note is that autophagy can have either direct or indirect contribution to protein secretion. For example, autophagy related proteins directly facilitate protein secretion by mediating cargo sequestration (as IL-1B) or vesicle trafficking (as insulin), but autophagy also maintains cellular homeostasis that is important to preserve the specialized function (IL-1B and vWF) and primes the plasma membrane for proper release of autophagy-independent secretory vesicle (as in osteoclasts). Furthermore, for sustaining a microbial barrier, autophagy's contribution is dual as both a canonical form of autophagy (xenophagy) and the regulation of important secretory factors as mucin contribute to prevent pathogen invasion.

Secretory autophagy: waste disposal?

Damaged and aggregated proteins and aged organelles are typically degraded by autophagy. Substrates for autophagy are ubiquitinated and recognized by autophagy receptors and degraded. Recent work indicates that defective or saturated autophagy, i.e. by defective autophagosome/ lysosome fusion results in cargo secretion into the extracellular environment.

Lysosomal dysfunction is associated with the secretion of aggregation prone proteins that are associated with neurodegenerative diseases as Parkinson's and Alzheimer's disease. α -Synuclein is a presynaptic neuronal protein that is genetically and neuropathologically linked to Parkinson's disease. Wild type α -synuclein is typically

degraded by the autophagy and the proteasome (36). Interestingly, tubulin polymerization-promoting protein/p25a, expressed in the CNS, sorts α -synuclein into autophagosomes but simultaneously prevents its degradation through inhibition of autophagosome/lysosome fusion. Instead p25a controls α -synuclein clearance by its release in the extracellular environment in an autophagy-dependent manner (37).

Alzheimer's disease is characterized by the accumulation of intracellular Amyloid beta (AB) peptide and tau aggregates and extracellular AB plaques. In normal conditions, intracellular proteins are cleared by autophagy and autophagosomes are resolved in the process. However, during Alzheimer disease, autophagosomes accumulate, indicative of impaired autophagy. Autophagy-deficiency (ATG7 knockout) in excitatory neurons results in intracellular AB accumulation, confirming its role in clearance of AB aggregates by autophagy. Although AB increased intracellularly, extracellular AB plaque formation was drastically reduced. Reconstitution of ATG7 expression by lentiviral transduction, rescued the secretory phenotype. In parallel, pharmacologic modulation by either induction or inhibition of autophagy, by rapamycin or spautin-1, increased and reduced extracellular AB release respectively. Thus, autophagy influences intracellular transport and secretion of AB (38).

Mitochondria, the energy producing centres of the cell, generate ROS as a by-product of oxidative phosphorylation. In many cancers, ROS production is increased due to mutations in mitochondrial DNA, hypoxia and a disturbed metabolism, leading to cancer progression(39). The homeostasis of mitochondrial ROS plays an important role in the regulation of autophagy. Depolarized and ROS leaking mitochondria are typically degraded by a selective form of autophagy, mitophagy. In depolarized mitochondria, PINK recruits Parkin to mediate selective removal of the organelle in a degradative autophagy-dependent manner. However, a recent report shows that there is an alternative way to maintain mitochondrial homeostasis in the cell. Mesenchymal stem cells (MSC) pack depolarized mitochondria in microvesicles and release them in the extracellular environment to outsource mitophagy where they are recognized by and transferred to macrophages. These released microvesicles are highly enriched in LC3 and ATG12 compared to whole-cell extracts. This mitochondrial transfer probably serves to increase MSC survival. (40). Similarly, LPS stimulated rat hepatocytes secrete mitochondrial proteins CPS1 and COXIV, a component of the mitochondrial respiratory chain and associated with the inner mitochondrial membrane, and mitophagy related proteins PARK2 and PINK1 and LC3B-II. These effects are inhibited by the autophagy inhibitor 3Methyladenine or after *Atg5* knockout. Suggesting a role for autophagy in the secretion of mitochondria after LPS stimulation (41).

Also endothelial cells are able to release vesicles with autophagosome characteristics. During apoptosis, endothelial cells release in addition to apoptotic bodies, vesicles in an unconventional manner(42). Ultrastructural analysis by electron microscopy showed single membrane vesicles up to 10 μ m which contained structures of mitochondria, multivesicular bodies and autophagosomes. Further proteomic analysis re-

vealed the release of autophagy associated proteins ATG16L1, LAMP2 and LC3B. The biological function of this phenomenon remains to be elucidated (43).

The previous section lists the evidence that cells are able to release autophagic vesicles into the extracellular environment. Vesicle release during defects in the autophagic process, specifically during autophagosome/ lysosome fusion, suggests alternative mechanisms in waste removal.

Effects on the tumour microenvironment

Regardless of the clinical advances in the past decades that have improved cancer patient outcome, cancer is still one of the leading causes of death in the world. Importantly, the efficacy of treatment strategies is heavily influenced by cancer cell autonomous features but also by the tumour microenvironment. Solid tumours consist of a variety of cell types, including the cancer cells, endothelial cells, immune cells, fibroblasts and contain well- and poorly perfused areas that results in inefficient nutrient and oxygen supply (44,45). Normal (non-transformed) cells in the tumour microenvironment (TME) are reprogrammed by the cancer cells to their benefit. This is exemplified by growth supporting angiogenesis and the suppression of anti tumour immunity. Importantly, an existing connection between autophagy and tumorigenesis has already been established. For example, deletion of a single *BECN1* allele (Beclin1 protein important regulator of autophagy (46)) predisposes mice to spontaneous tumour development (47,48). Further, depletion of FIP200 (important for autophagy initiation) in mammary cancer cells inhibits tumour initiation and progression including metastases (49). Autophagy in cancer cells supports their survival (by aiding the high energy demand) and abets resistance to metabolic and oxidative stresses (e.g. hypoxia) (50-55). Although this role is well established, the contribution of autophagy related intercellular communication that influences tumour progression through evasion of immunosurveillance, immunogenic cell death, angiogenesis and an invasive phenotype is an emerging field with great interest. For example, in cancer the RAS genes HRAS and KRAS are frequently mutated. Although the exact role of autophagy in tumour progression of RAS mutated tumours is still under debate, autophagy seems to be dispensable for the growth and survival of KRAS mutated cancer cell lines derived from human tumours (56). However when non-RAS mutated cells are transformed with oncogenic RAS, these cells are highly dependent on autophagy for tumorigenic events (57). In addition, the invasive phenotype of HRASV12-transformed breast cancer cells is reduced in ATG7 knockdown cells. This invasive phenotype could be rescued by incubating these autophagy deficient cells with conditioned medium of autophagy proficient cells. This supports an autophagy-dependent secretory system that supports tumour progression (58).

In the next section, the current knowledge on proteins secreted through autophagy-mediated processes that influence tumour progression is discussed (table 2).

1. Influencing immunogenic cell death to evade immunosurveillance

Under normal circumstances, immune cells (including dendritic cells (DCs), natural killer (NK) cells and T cells) recognize and eliminate newly formed neoplastic cells due to their high immunogenic nature as a result of their mutational burden (immunosurveillance). Cancer cells that have obtained an immune evasive phenotype are able to circumvent recognition and subsequent elimination by the cooperative immune cells. These cancer cells are then selected for characteristics that circumvent local immunosurveillance and contribute to the growth of the lesion. With recent advances, the immune evasive feature is a topic of interest for the development of therapeutic strategies. Ideally, the elicited cancer therapy-induced cell death should provoke an immunogenic chain reaction that includes boosting the immune system to tilt the balance towards recognition rather than evasion, called immunogenic cell death (ICD). ICD invokes the release of immunomodulatory proteins (damage-associated molecular patterns (DAMPs) that incite anti tumour immunity (59). ICD can be induced by selective chemotherapeutics including mitoxantrone (MTX) and oxaliplatin (OXA) and radiotherapy (reviewed in (60)). Cancer cells undergoing ICD stimulate and activate the innate immune cells. Subsequently this can result in the cross-priming of the adaptive immune system for the antigens of dying cancer cells, thereby leading to an effective activation of anti tumour immunity. This can elicit a long-term therapeutic effect (even after therapy has stopped) and is fundamental to observed abscopal effects. In line, clinical studies have demonstrated that lymphopenia negatively affects chemotherapeutic response of solid tumours (61) and that ICD-associated DAMPs can be used as predictive and prognostic biomarkers (62).

Important immunogenic DAMPs that are displayed by cells undergoing ICD are secretion of ATP, surface exposure of calreticulin (CRT), release of heat shock proteins and high mobility group box 1 (HMGB1). Interestingly, ATG5 knockdown in colon cancer cells reduces ATP release after radiotherapy (63) and MTX exposure (64), which was associated with a decreased effect on tumour growth inhibition. Interestingly, in autophagy deficient cells no differences in CRT surface exposure or HMGB1 release were observed (64). The relevance of autophagy-dependent radiotherapy-induced ATP release was further supported by the observation that treatment with an inhibitor of ecto-ATPase only increased radiosensitivity in immunocompetent but not immunodeficient mice. Here, a partial rescue of lymphocyte infiltration indicates that the autophagy-dependent radiotherapy-induced ATP release enhances anti-tumour immunity (63). Similarly, for MTX treatment of osteosarcoma cells it was also shown that ATG5 is required for ATP secretion. Interestingly, on a more mechanistic level, Martins et al demonstrated that ATP (stored in lysosomes) is released upon MTX and OXA treatment and is associated with LAMP1 (lysosomal marker) translocation to the plasma membrane. Nonetheless, a role for autophagy seems to be maintaining an intracellular ATP pool (may even be cargo sequestration) required for (LAMP1+) lysosome-dependent ATP release. Accordingly, the replenishment of ATP to lysosomes was reduced when

autophagy genes were knocked down (65). ATP release conveys an important “eat me” signal for immune cells. Once it is released, ATP may attract innate effector cells of the immune system into the tumour bed. Consistently, it was demonstrated that autophagy-dependent ATP release from MTX treated colon cancers was a stimulus for DC recruitment, IFN γ -producing CD4 and CD8 T cells that had favourable effects on MTX sensitivity (64). Correspondingly, ATG7 in a genetically-induced melanoma mouse model was required for MTX-dependent growth inhibition that was reliant on functional CD4 and CD8 T cells (66). Further, caloric restriction or treatment with caloric restriction mimetics, that increase autophagy activity, enhance autophagy-dependent ATP release and improve MTX-induced tumour growth delay in a T cell dependent fashion (67).

Oppositely, a different study using a different ICD inducer (photo-oxidative ER stress inducer hypericin) has demonstrated autophagy-independent ATP release, but observed enhanced surface CRT exposure when autophagy was attenuated. The enhanced DC maturation and IL-6 secretion further promote IFN γ producing T lymphocytes (68). Interestingly, surface CRT exposure after MTX or hypericin treatment could be ablated in cells lacking lysosome associated LAMP2A, an essential gene for a chaperone-mediated autophagy (69). Moreover, regarding ATP it was demonstrated that extracellular residing ATP does not relay an immunogenic response *per se* (70) and further illustrates the context dependence of effects resulting in immunogenicity.

The involvement of autophagy-related proteins in the release of the DAMP HMGB1 has been demonstrated using ATG5^{fl/fl} Cre⁺ bone marrow-derived macrophages (11) and dying glioma cancer cells (in which HMGB1 was found in a subset of autophagosomes before release (71)). The released HMGB1 by dying cancer cells can bind Toll-like receptor (TLR)-4 and promotes the processing and presentation of tumour antigens by DCs. This leads to cross-priming of T-cells and enhances immunosurveillance (72). Furthermore, endothelial cell exposure to HMGB1 triggers pro-angiogenic effects (73) including endothelial cell migration, sprouting and induction of an autocrine signalling cascade that results in elevated expression of leukocyte adhesion molecules ICAM-1, VCAM-1 and E-selectin. Moreover, HMGB1 induced expression of VEGF-A, VEGFR1, VEGFR2 and neuropilin-1 (74) and stimulation of angiogenesis (75).

In conclusion, ICD is an important pillar of therapy-induced anti-tumour immunity as it relays important signalling to the immune system, including DCs. DC stimulation may be important to induce tumour cure as demonstrated by ICD-based DC-vaccines in high grade glioma bearing mice (76). Nevertheless, the influence of autophagy on the display of DAMPs may be ICD-inducer dependent and requires further understanding for effective use.

II. Cytokine release and influence on the tumour microenvironment

ATP can bind the P2RX7 receptor and activate the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in DCs and macrophages (77). This inflam-

masome activation can be suppressed by autophagy (20), which would be favorable as inflammasome activation and subsequent IL-1B release by e.g. macrophages have pro-tumorigenic effects. However, the P2RX7 receptor impairs autophagy by blocking lysosomal function and stimulates release of vesicles with autophagolysosome characteristics (78). This suggests that activation of the P2RX7 receptor leads to a secretory phenotype, via inhibition of autophagy. Depletion of IL-1B arrests growth in melanoma (79) and macrophage-derived IL-1B induced IL-17 expression from $\gamma\delta$ T cells resulted in expansion of tumour associated neutrophils that suppress cytotoxic T cells in breast cancer, resulting in increased number of pulmonary and lymph node metastases (80). Alternatively, in a murine colon carcinoma model autophagy-dependent ATP release after MTX treatment promoted recruitment of IFN γ -producing CD8 $^{+}$ T cells into the tumour in an IL-1B-mediated fashion (64).

Lipopolysaccharide (LPS)/LPS+ATP stimulated GABARAP Knockout macrophages to secrete more IL-1B and IL-6. In addition, GABARAP knockout lymphocytes produced more IL-2 and interferon- γ . In this model, GABARAP KO was associated with reduced tumour incidence. These effects were validated on tumour control in a melanoma tumour cell inoculated model (81) and indicate that GABARAP in non-cancerous cells is sufficient to sustain pro-tumorigenic effects potentially due to control of cytokine secretion.

A study using MMTV-PyMT mouse model of breast cancer-bearing a conditional deletion of autophagy gene *FIP200* shows that these tumour cells have a different chemokine secretion profile than FIP200 proficient cells. The TME polarizes towards an improved immunosurveillance as enhanced secretion of chemokines including CXCL9, CXCL10 and CXCL11 lead to increased infiltration of IFN γ -producing CD8 $^{+}$ and CD4 $^{+}$ T cells (49).

In conclusion, autophagy related secretion is important in controlling the cytokine profile of different cell types.

III.a. Pro-metastatic: Driving an invasive behavior of cancer cells

Oncogenic mutations in RAS are highly prevalent in cancers and drive different pro-tumourigenic features including proliferation, survival and invasion. Autophagy deficient HRAS^{V12} transformed breast cancer cell lines display reduced invasive protrusions in genetic knockdown models (including ATG7, ATG12 and ATG3) and after pharmacological inhibition (chloroquine or bafilomycin A1). Addition of conditioned medium of autophagy-proficient cells rescues the invasive phenotype, indicating a role for autophagy-dependent secretion in triggering cellular migration. Correspondingly, the pulmonary metastatic potential of HRAS^{V12} tumours is reduced in autophagy deficient cells, effects dependent on autophagy-related secretion (58). Despite these results in this model, the role of IL-6 in tumour progression is ambiguous in literature as both pro-tumorigenic

(metastasis, angiogenesis, immune suppression) and anti-tumorigenic (CD8+ T cell trafficking to lymph nodes and tumours) effects on the TME are described (82).

Kraya et al observed a cytokine profile that differed between melanoma cells with high and low autophagy activation that could be mimicked by introducing ATG7 knock-down in an autophagy high cell line. The main secretory factors the authors focused on that were dependent on autophagy(-protein) are, leukemia inhibitory factor (LIF), family with sequence similarity 3 member C (FAM3C), dickkopf WNT signalling pathway inhibitor 3 (DKK3) and IL-8. These factors are able to promote metastasis via mechanisms that include MMP2 upregulation (IL-8), epithelial to mesenchymal transition (FAM3C, DKK3, LIF) and promotion of angiogenesis (IL-8 and DKK3) (83).

TLR3 and TLR4, that are expressed on immune cells including macrophages and DCs but also a variety of cancer cell, can activate the release of an array of cytokines (84). Recently, it has been demonstrated that TLR3 and TLR4 activation (in an LPS or poly(I:C) induced model) in lung cancer cell lines (A549 and H460) causes 1) Lys63 linked ubiquitylation of TNF receptor associated factor 6 (TRAF6) and 2) induced autophagy. Herein, autophagy was required for TRAF6 ubiquitination that leads to downstream activation of NFkB and MAPK signalling and subsequent cytokine production. As a result, autophagy deficiency in these lung cancer cells reduced release of IL-6, C-C motif chemokine ligand (CCL)-2, and CCL-20. CCL-2 secretion is associated with cell migration and CCL-20 with a metastatic phenotype. Accordingly, autophagy deficiency impaired migratory capacity. IL-6 can induce VEGFA and MMP release that are associated with an invasive phenotype. Indeed, the invasive phenotype was dependent on autophagy and IL-6 and associated with VEGFA and MMP2 release (85).

In breast cancer autophagy inhibition through ATG7 or Beclin1 knockdown altered IL-6 secretion. Interestingly, autophagy deficiency increased IL-6 secretion by MCF7 (low autophagy-dependent survival) and decreased IL-6 secretion by MDA-MB-468 (high autophagy-dependent survival) cells. IL-6 secretion is important for cancer stem cell (CSC) maintenance and is sufficient to induce CD44⁺:CD24^{low/-} phenotype in breast cancer cells (86). In line, autophagy deficiency decreased mammosphere formation capacity of MDA-MB-468 cells. Rescue experiments illustrated that mammosphere formation was improved by IL-6 treatment and conditioned media from autophagy proficient MDA-MB-468 cells. Autophagy-dependent secretion of IL-6, but also other factors, are able to promote mammosphere formation and may be important in CSC maintenance (87).

III.b. Pro-metastatic: Weibel-Palade body formation in vascular endothelium to facilitate metastasis

Aberrant signaling in tumour-associated endothelial cells contributes to excessive neo-vascularization that is a feature of solid tumours (reviewed in (75)). As discussed above, autophagy is important to sustain the secretion of protein-containing WPB by proper

vWF maturation. In tumour endothelial cells (TECs) these WPBs contain important secretory factors that can influence tumour progression. In line, P-selectin is sorted into WPBs as a result of its ability to interact with vWF and is translocated to the cell membrane upon stimulation (88). Once localized at the luminal side of the endothelial cell, it facilitates metastasis formation by promoting adhesion of circulating tumour cells (89). Impairment of autophagy may therefore reduce development of metastases. In addition to vWF and P-selectin, WPBs can contain other secretory proteins as angiopoietin 2 which is positively associated with tumour progression (due to its angiogenic potential) and interleukin 8 which is important in tumour progression and metastasis (due to its angiogenic and immune response modulating potential). Collectively, autophagy-dependent WPB formation may facilitate tumour progression, although these aspects require further investigation.

IV. Therapy resistance

Autophagy has been implicated in promoting chemo- and radioresistance. Although often presumed to be caused by its degradative feature, we demonstrated in irradiated cancer cells that knockdown of ATG7 or LC3B, but not treatment with lysosomal inhibitor chloroquine, sensitizes cancer cells to radiation (90). This further supports a role of autophagy-related proteins to promote radioresistance through an alternative process such as secretion. For example, HMGB1 is secreted through autophagy-dependent mechanisms during ICD (11,71). Although this factor is an important DAMP that can increase immunogenic responses, HMGB1 increases Doxorubicin resistance in neighboring breast cancer cells (91). In line, the interaction of HMGB1 with the receptor for advanced glycation end products (RAGE) that is expressed on various cell lines in the tumour increases chemo resistance by inducing pro-survival autophagy (92).

In addition to increasing angiogenesis and the pro-metastatic potential by DKK3 DKK3 expression is associated with docetaxel chemo sensitivity in lung cancer cells through decreasing expression of the drug efflux pump P-glycoprotein (93). Furthermore, DKK3 overexpression in an esophageal adenocarcinoma cell line was associated with increased 5-FU and cisplatin resistance, invasion and activation of the TGF- β signaling (94).

In short, autophagy-dependent secretion is involved in anti-tumour effects through enhancing immunosurveillance, but is also important in tumour progression through stimulation of angiogenesis, changing drug resistance, triggering EMT and increasing metastases development. Manipulation of the secreted arsenal of proteins, and tilting the balance more towards an anti-tumour strategy may be an attractive novel approach in cancer treatment.

Table 2 autophagy dependent secreted factors

Protein	Mechanism	REF
Acbp	Atg5, Atg7, Atg8, Atg12	(16)
IL-1B	ATG5	(11)
NBR1, P62, OPTN and CACO2 LC3B-II	Inhibition or depletion of PIKfyve by apilimod or siRNA	(19)
angiogenin 4, interlectin 1 and relmβ	ATG16L1 ATG5 or ATG7	(25)
MUC5AC	ATG5 and ATG16L1	(26)
Insulin	GABARAP	
NPY	ATG16L1 as RAB33a effector	(33)
von Willebrand factor (vWF), P-selectin, interleukin-8, angiopoietin-2 and endothelin-1	ATG7,ATG5, chloroquine or bafilomycin A1	(34)
matrix-degrading factors including cathepsin K	ATG5 or ATG7	(35)

Autophagy-dependent receptor trafficking in tumour progression

Autophagy execution requires cargo recognition, packaging, vesicle transport, vesicle fusion and degradation. In addition to the catabolic function of autophagy, the autophagy machinery is utilized for more purposes including intracellular trafficking and endocytic signaling. In addition to these roles in secretion, autophagy mediates the retro- and anterograde trafficking of membrane bound receptors that may influence tumour progression.

For example, the GABARAP protein family mediates membrane of cell- surface expression of receptors like the GABA (A) receptor (GABAAR (95), the human kappa opioid receptor (hKOPR) (96), transient receptor potential cation channel subfamily V member 1 (TRPV1) (97), the angiotensin II receptors (AGTR) (98,99) and the epidermal growth factor receptor (EGFR) (100).

EGFR controls cell proliferation, migration and differentiation and is frequently over activated in several cancer types due to amplification or mutation (101). EGFR expressing tumours depend on autophagy for their survival and proliferation. Inhibition of autophagy by the administration of chloroquine abrogated the radioresistant phenotype of these tumours (55)(reviewed in (102)). Interestingly, during hypoxia translocation of EGFR to the plasma membrane is controlled by GABARAPL1 (100). Upon hypoxia exposure, GABARAPL1 colocalizes with EGFR at the cytoplasmic site of the plasma membrane. Moreover, knockdown of GABARAPL1 resulted in a decrease in EGFR membrane expression, but not in overall EGFR expression, suggesting a role for GABARAPL1 in anterograde transport of EGFR.

The KOR and GABAAR are involved in neurological processes and play a role in a variety of processes like pain sensation, consciousness, and mood.

The GABA(A) receptor (GABAAR) is well known for its inhibitory role on active neurons and is expressed on the postsynaptic throughout the whole body, although mainly expressed in the mammalian brain. Surprisingly, overexpression of the GABAAR leads to

several types of cancer including breast, liver, lung and pancreatic cancers and contributes to migration of breast cancer cells through activation of extracellular-regulated kinase 1/2 (ERK1/2) (103-108). GABARAP and GABARAPL1 are both involved in GABAAR trafficking towards the plasma membrane. In this role, GABARAP probably serves as a cargo-receptor which mediates GABAAR incorporation in the transport vesicle by a direct interaction with the $\gamma 2$ subunit of the receptor. GABARAP knockdown mice show no defects in GABAAR expression, suggesting that GABARAP is redundant and other molecules, like its homolog GABARAPL1, can take over its function (109).

In contrast to the pro-tumorigenic effects of EGFR and GABAAR signalling, membrane expression mediated through autophagy-related proteins also results in the expression of receptors that may inhibit tumour progression, for example through KOR signalling. The KOR is well characterized for its analgesic role. However, the KOR also acts as a negative regulator of cell proliferation in breast, lung and prostate cancers (110,111). Opioid receptors belong to the GPCR family, and activation of the receptors modulates the MAPK pathway and inhibit pro survival PI3K/AKT signalling molecules and may antagonize EGFR signalling (110). Both GABARAP and GABARAPL1 are required for anterograde transport of the KOR receptor. Interestingly, because of its stronger interaction GABARAPL1 does not need C-terminal modification in contrast to GABARAP, which requires membrane association to transport the KOR to the plasma membrane (96).

Taken together, the GABARAP family proteins mediates trafficking and surface expression of receptors with both tumour promoting (EGFR, GABAAR) and tumour inhibitory characteristics (KOR). This suggests that the GABARAP family contributes to cancer progression in a context-dependent manner, being in a tumour promoting or inhibitory role.

Concluding remarks

Autophagy has been considered as an important tumour suppressive process for cellular homeostasis by effectuating lysosomal degradation of the cells' toxic constituents. Importantly autophagy mediates an additional cellular feature, the trafficking and release of specific proteins. These effects are important during physiological conditions (e.g. maintaining a barrier for infection by mucus and lysozyme secretion and waste secretion), but also mediate important effects in tumour progression (figure 1). The autophagy mediated secreted factors may stimulate cellular proliferation via auto- and paracrine signaling and establish a communicative tool between cells that can either stimulate or limit tumour progression. The autophagy mediated release of DAMPs seem to be ICD inducer-dependent and polarize the tumour microenvironment towards a less immunosuppressive phenotype. Alternatively, tumours are characterized by promoting an immunosuppressed tumour microenvironment by cytokine signaling. Furthermore, autophagy mediated secretory signaling promotes an invasive phenotype.

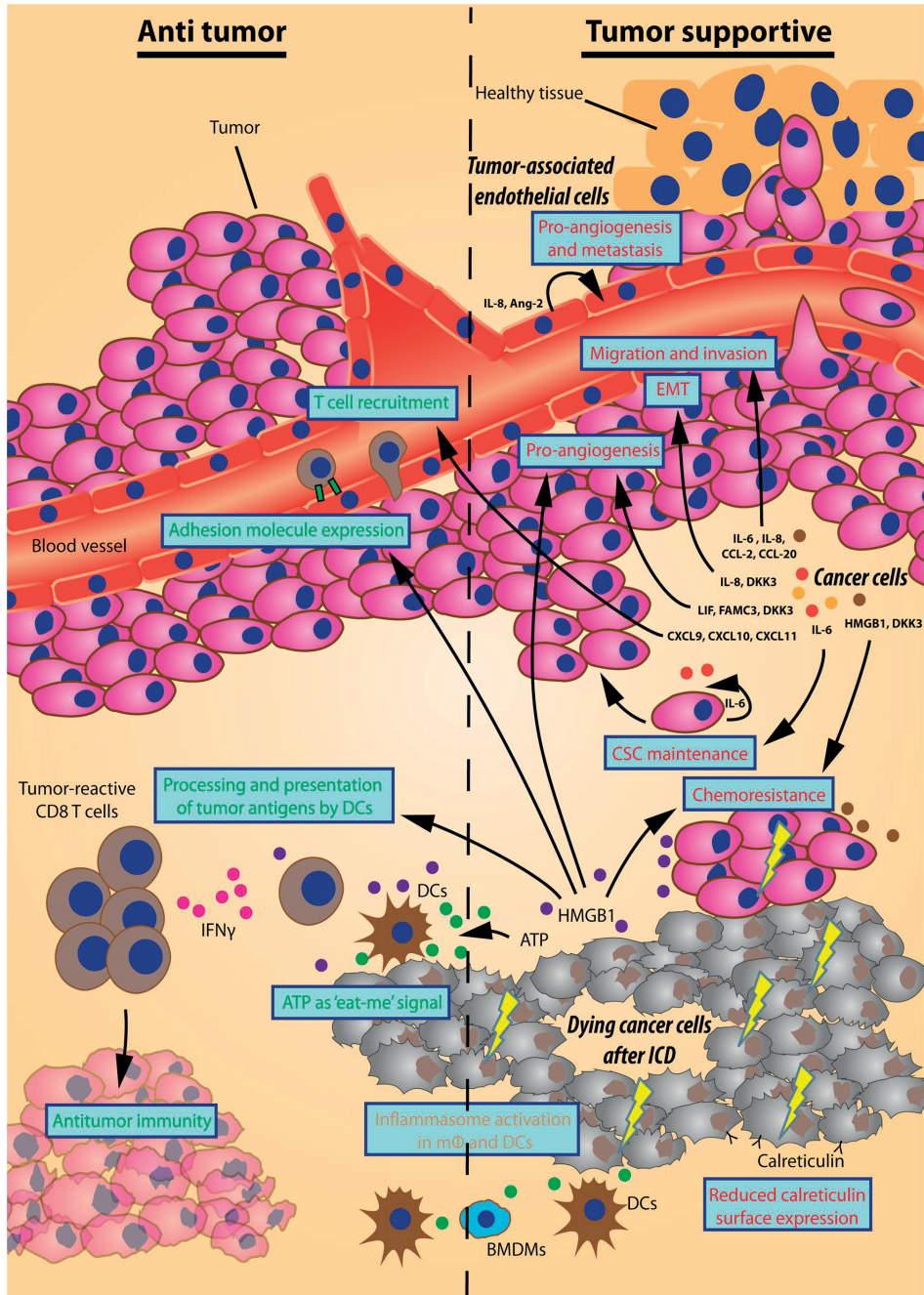


Figure 1. Effects of autophagy dependent secretion on tumour progression.

Schematic representation of autophagy mediated secretory events that either inhibit or support tumour progression displayed here on the left ('Anti tumour') and right side ('Tumour supportive') respectively. The different sources of autophagy dependent secretory factors (***bold italic***) establish multiple effects on the

tumour microenvironment (in blue boxes) by the designated factors. As such, factors that promote angiogenesis, invasion, a migratory phenotype, cancer stem cell (CSC) maintenance or chemoresistance support tumour progression. Also, a reduced surface expression of calreticulin by cancer cells undergoing immunogenic cell death (ICD) hinders an immunogenic response. Oppositely, some factors have counteractive effects on tumour progression by improving immune cell adhesion or recruitment. Moreover, the 'eat-me' signal ATP together with HMGB1/TLR4 mediated improved processing and presentation of tumour antigens by dendritic cells (DCs) promote interferon gamma (IFN γ) producing T cells to aid antitumor immunity. The ATP/P2RX7-mediated activation of the inflammasome in macrophages (m ϕ) and DCs can have an array of effects of which the final inhibition/support of tumour progression may be context-dependent.

An important note is that autophagy may convey direct or indirect effects on secretory events through formation of the mobilized vesicle, facilitation of anterograde trafficking or alterations in homeostasis and/or autonomous cell signaling.

In conclusion, autophagy (or autophagy-related proteins) is an important cellular process that is more elaborate than solely a degradative pathway. It facilitates multiple secretory events that can promote tumour progression by limiting immunosurveillance and stimulating invasiveness and angiogenesis.

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Chapter

7

GABARAPL1 is required for the secretion of pro-angiogenic extracellular vesicles during hypoxia

Tom G. Keulers¹, Sten F. Libregts², Marco B.E. Schaaf¹, Hanneke J.M. Peeters¹, Kim G. Savelkoul¹, Johan Bussink³, Hans Duimel⁴, Marijke I. Zonneveld¹, Karel Bezstarosti⁵, Jeroen A. Demmers⁵, Marc Vooijs¹, Marca Wauben², Kasper M.A. Rouschop¹

¹Maastricht Radiation Oncology (MaastRO) lab, GROW – School for Oncology and Developmental Biology, Maastricht ²University, Maastricht, the Netherlands ²Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, ³Utrecht University, Utrecht, Netherlands. ³Department of Radiation Oncology, Radboud University Medical Center, ⁴Nijmegen, the Netherlands. ⁴ Maastricht Multimodal Molecular Imaging Institute, Division of Nanoscopy, University of ⁵Maastricht, the Netherlands. ⁵Proteomics Center, Erasmus University Medical Center, Rotterdam, the Netherlands.

Chapter 8

GABARAPL1 is essential for metastasis formation in mice

Tom G. Keulers¹, Imke Demers¹, Sten Libregts², Kim Savelkouls¹, Joris J. Roelofs³, Johan Bussink⁴, Marijke I. Zonneveld¹, Marca Wauben², Kasper M. Rouschop¹

¹Department of Radiotherapy, GROW - School for Oncology and Developmental Biology, Maastricht University Medical Centre+, Maastricht, The Netherlands

²Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

³Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

⁴Department of Radiation Oncology, Radboud University Medical Center, Nijmegen, The Netherlands.

Chapter 9

Summary and general discussion

Cancer is still causing great morbidity and mortality worldwide and as our population ages, the incidence rates are increasing. Cancer arises due to genetic changes that accumulate over cellular generations and cancer cells are selected to withstand the sometimes harsh conditions observed within the tumour. In addition, cancer cells use the same general mechanisms that normal cells utilize to adapt to (micro-) environmental changes.

Tumours are characterized by the presence of immature and often chaotic vasculature that results in insufficient blood supply to areas within the tumour. The high metabolic rate of cancer cells in combination with insufficient oxygen delivery results in hypoxic tumour regions, a common feature of most solid tumours. Oxygenation in tumours may vary from normal values (~5%) close to the vessel wall to complete anoxia (0% O₂) close to perinecrotic regions. Hypoxic cells are more resistant to chemo- and radiotherapy and are associated with increased metastasis resulting in poor prognosis of patients. Although common in tumours, hypoxia (especially severe hypoxia) is not observed in the human body and therefore poses a therapeutic opportunity. Fundamental understanding of how cancer adapts to hypoxic episodes is therefore essential to develop novel therapeutic strategies. The work in this thesis addresses novel findings how cancer cells respond to hypoxia. One of the mechanisms that allows cells to survive hypoxia is autophagy, consequently autophagy inhibition increases therapy efficacy in pre-clinical studies and cancer patients (1,2) (+ **chapter 4**). Autophagy is a mechanism that allows the cell to recycle aged or obsolete subcellular compartments/ organelles and protein aggregates to maintain cellular homeostasis. During normal conditions the autophagic rate is relatively low, but can be rapidly upregulated during periods of (metabolic) stress. In cancer cells elevated autophagy-activity is frequently observed. Autophagy is dependent on the formation of intracellular vesicles, the autophagosome. The cargo to be degraded is randomly or selectively sorted into these autophagosomes. The latter via specific adaptor proteins that recognize ubiquitylated substrates. These proteins act as an adaptor between the recognized cargo and members of the LC3/GABARAP protein family. This LC3/GABARAP protein family consists of 7 members including LC3A, -B and C, and GABARAP, GABARAPL1 and GABARAPL2, which share a high degree of homology in amino acid sequence and tertiary structure. The last decade most of autophagy research has been focused on LC3B. Yet, due to the high degree of homology and lack of unstructured overview the family members' function, the current dogma is that all LC3B/GABARAP protein family members are redundant and functionally involved in autophagy. In **chapter 2**, we systematically reviewed the current knowledge on the individual functional role of the LC3/ GABARAP protein family members. In addition to overlapping functions, most LC3/ GABARAP protein family members mediate unique, autophagy (in)dependent functions. Proteins from the GABARAP subfamily display several functions in intracellular trafficking. For example, GABARAP and GABARAPL1 are required for the trafficking and membrane expression of receptors, including the kappa opioid receptor, the angiotensin receptor II and the GABA receptor

(3-5), but also the transient receptor potential cation channel subfamily V member (TRPV). It is still unclear how these processes work exactly, but it is suggested that they might function as a scaffold protein between kinesin motor proteins and the transport vesicle (6). GABARAPL1 is also shown to interact with motor proteins, however the functional role is still under investigation. In addition, we identified a role for GABARAPL1 in membrane expression of the epidermal growth factor receptor (EGFR) during hypoxia (**chapter 3**). Interestingly, GABARAPL1 expression is induced during hypoxia, which suggests a specific function during these conditions. The upregulation is dependent on increased transcription orchestrated by the PERK-ATF4 arm of the unfolded protein response (UPR), a cellular stress responsive pathway that senses unfolded and misfolded proteins in the ER. Furthermore we observed that only GABARAPL1, and not GABARAPL2 mRNA expression was induced during hypoxia, indicating unique regulatory mechanisms that control expression and suggesting that both proteins are required to fulfill specific functions during these conditions.

Tumour hypoxia is an independent prognostic marker for treatment outcome, irrespective of the treatment modality. Because GABARAPL1 expression is induced during hypoxia, we tested if GABARAPL1 expression could be linked to treatment outcome. In a panel of head and neck cancer patients GABARAPL1 expression correlated with reduced local and loco-regional control of these patients. In addition to the physical presence of hypoxia that contributes to resistance to therapies, GABARAPL1-dependent membrane expression of tumour growth stimulating receptors, such as the EGFR, and the secretion of pro-angiogenic factors (**chapter 7**) can contribute to decreased treatment outcome. In contrast to our studies, other groups reported tumour suppressive functions of GABARAPL1 and correlated GABARAPL1 expression to increased survival (7). However, these studies addressed different tumour sites, suggesting tumour specific mechanisms. Summarizing, this suggests that GABARAPL1 expression could be linked to treatment outcome and potentially could be used as a prognostic biomarker.

In addition to their role in autophagy and intracellular trafficking of receptors, LC3/GABARAP protein family members but also other proteins of the core autophagy machinery, have been associated to secretory mechanisms. In **chapter 6** we summarized current knowledge about autophagy-dependent secretion and what the consequences are on tumour progression. In the classical ER-to-Golgi route, proteins are marked for secretion by a leader-peptide, which serves as a secretion signal. However, also proteins without this sequence can be secreted via alternative forms of unconventional secretion in an autophagy-dependent manner (8-10). Autophagy-dependent secreted factors are actively released and have pro-metastatic properties can stimulate proliferation via auto- and paracrine signalling but also have immune modulating properties that can promote the invasive phenotype.

Also defects in autophagy, such as defects in lysosome/ autophagosome fusion, can lead to secretion of the cargo in the extracellular environment. The concept autophagy-dependent secretion is still in its infancy and is poorly understood. Many questions

remain unanswered: How is the autophagy machinery involved in the biogenesis of secretory vesicles/ exosomes? What differentiates secretory vesicles from degradative vesicles? Where do these machineries converge? What is the signal for secretion?

After molecules have been internalized through several mechanisms of endocytosis, the internalized cargo are sorted in vesicular compartments referred to as endosomes after which they can have several different fates. Endosomes can be degraded through fusion with autophagosomes, or directly via fusion with lysosomes, but they can also be recycled back to the plasma membrane via formation of multivesicular bodies (MVB) which arise after intraluminal vesicles (ILV) formation by inward budding of the endosomal membrane. At this point, different sorting mechanisms, such as the ESCRT machinery are involved in fine-tuning the sorted cargo into these ILVs. Once MVBs fuse with the plasma membrane, ILVs are released in the extracellular environment as exosomes and are considered as extracellular vesicles.

Extracellular vesicles are emerging as vehicles of intercellular communication and more is known about their role in physiological and pathological conditions such as cancer. EVs are small membranous particles which are secreted by most, if not all cells and contain biological information such as miRNAs and proteins. In general, EV content seems to reflect the status of the cell of origin, however, accumulating evidence shows that specific molecules are enriched in EV subpopulations. **In chapter 7**, we demonstrate that during severe hypoxia, cells secrete a subpopulation of EVs that are characterized by a difference in size. This subpopulation is secreted in a GABARAPL1 dependent manner. These EVs, which are marked by GABARAPL1 expression on EV membrane, have pro-angiogenic properties that stimulate tumour growth. Depletion of GABARAPL1 through the expression of shRNAs alters EV composition in a cell line dependent manner. Xenografts of these GABARAPL1 knockdown cells displayed reduced growth, reduced vessel density and increased necrosis. However, the effect of GABARAPL1 depletion on EV secretion and composition seems to be cell line dependent. In HT29, GABARAPL1 depletion leads to decreased marker expression and decreased total EV counts. In contrast, in U87, GABARAPL1 depletion leads to decreased expression of some markers, but an increase in the tetraspanin CD63. Also total secreted EV counts were not affected. GABARAPL1 deficient MDA-MB-231 cells (**chapter 8**) show an increase in the tested markers CD81 and CD63 and differences in RNA loading. Currently, the mechanism how GABARAPL1 participates in EV secretion is under investigation. However, current data suggests that GABARAPL1 is involved in sorting of EV cargo rather than transport. This is supported by the observation that GABARAPL1 deficient cells have enlarged endosomal (CD63+) compartments (presumable late endosomes/ MVBs), which indicates defects in endosomal pathways. In HT29 cells, this sorting mechanism is probably disturbed to such an extensive degree, that also total EV secretion is decreased.

LC3/ GABARAP subfamily members can be conjugated to phosphatidylethanolamine (PE) at the C-terminal site to associate with membranes. For LC3 subfamily members,

this conjugation is required for autophagy. GABARAPL1 also contains this C-terminal glycine, but this site is not required for GABARAPL1 mediated EV secretion (**chapter 7**). That GABARAPL1 is membrane bound, we demonstrated with cryo-transmission electron microscopy experiments, where we showed that after secretion, GABARAPL1 expression is retained at the EV surface. This might suggest that other post-translational mechanisms are at play to bind GABARAPL1 to the EV membrane. How this modification works and if this requires modification at the N or C terminal site, remains to be elucidated.

That GABARAPL1 expression is retained at the EV surface can be of therapeutic interest. In a small group of patients with glioblastoma, colorectal cancer and small cell lung cancer we detected elevated levels of GABARAPL1⁺EVs in plasma compared to healthy volunteers. As we demonstrated that GABARAPL1⁺EVs are secreted by hypoxic cells and are functionally active by stimulating angiogenesis and potentially mediating metastasis formation, this means too they could be explored as a therapeutic target or as biomarkers to determine the hypoxic levels of a tumour. That inhibition of GABARAPL1⁺EVs works to inhibit blood vessel formation we showed in vitro by preventing GABARAPL1⁺EV function with blocking antibodies to inhibit tube formation. Currently we are developing monoclonal antibodies to explore the therapeutical potential in vivo. In these studies we will explore tumour growth inhibition and prevention of metastasis. Furthermore, we will explore to potential of GABARAPL1⁺EVs as a biomarker to determine the hypoxic fraction of a tumour. Additionally, methods to determine GABARAPL1⁺EVs in the blood of cancer patients are being developed.

In addition to pro-angiogenic properties of GABARAPL1⁺EV, GABARAPL1⁺EVs are potentially involved in metastasis formation (**chapter 8**). We observed a decrease of 80% in lung metastasis in GABARAPL1 deficient orthotopically implanted breast tumours in mice, indicating an important role for GABARAPL1 in mediating metastasis formation. Metastasis is complex mechanisms where the cell require survival of multiple stages in the cascade. To develop into a metastasis, cells need to detach from the tumour, enter and survive the circulation and infiltrate the tissue and outgrow to a metastasis. Previously, we showed that GABARAPL1⁺EV have growth stimulating properties at distant tumour sites, we were therefore prompted to investigate the role of GABARAPL1 in metastasis development. Although this remains work in progress to elucidate the role of GABARAPL1 in metastasis formation, several steps in the metastatic cascade were addressed. GABARAPL1 seems to contribute to metastasis formation by development of the pre-metastatic niche. During development of the pre-metastatic niche, the micro milieu of the distant tissue is prepared in such a way that circulating tumour cells can colonize. To show this, we implanted breast tumours orthotopically in the fat pad of mice, consisting of a mixed population of Green fluorescent protein (GFP) expressing control cells, and Red fluorescent protein (RFP) expressing GABARAPL1 knockdown cells. After the tumour reached the maximum size, lungs were removed and lungs were examined for green and red fluorescent metastases. If the decrease in metastasis would

be caused by cell intrinsic changes caused by GABARAPL1 knock down, such as decreased membrane expression of certain receptors, we expect to identify GFP expressing metastases only. If the decrease in metastases was caused by a defect in tumour derived secreted factors, such as EVs, we expected to identify control and GABARAPL1 deficient metastases in comparable numbers. In the lungs we observed control cell-derived metastases as well as GABARAPL1 knockdown derived metastases, which supports the hypothesis of a GABARAPL1 mediated pre-metastatic niche formation. Interestingly, RFP expressing GABARAPL1 knockdown cells were always accompanied by GFP expressing control cells, which also could suggest a metastatic niche formation by the control cells, rather than a pre-metastatic niche. Taken together we can conclude that GABARAPL1 is important for metastasis formation, and that this is potentially mediated through GABARAPL1⁺EV.

Epidermal growth factor receptor (EGFR) is a member of ErbB family of receptors and is expressed in normal tissues where it is involved in processes as proliferation and inhibition of apoptosis (11). Mutation or over expression of the receptor can lead to several pathologies such as Alzheimer and cancer. EGFRvIII is a mutated form of EGFR characterized by a deletion of exon 2- 7 of the extracellular domain and is most prevalent in glioblastoma multiforme patients (GBM) who have a very poor prognosis (12,13). As a consequence of deletion of the extracellular domain the receptor is constitutively active, promoting pro-oncogenic effects and tumourigenicity (14,15). We demonstrated that EGFRvIII expressing tumours display increased activation and dependency on autophagy during metabolic stress including hypoxia. Typically EGFRvIII⁺ tumours show an increased tolerance towards hypoxia resulting in an increased hypoxic fraction. Additionally, EGFRvIII⁺ tumours show increased growth and increased regrowth rates after radiotherapy, resulting in decreased treatment outcome. Tumour hypoxia is a major limiting factor in cancer treatment (16). Therefore, targeting hypoxia in these tumours may contribute to increased survival rates. We demonstrate that EGFRvIII⁺ cells are more dependent on autophagy during hypoxia than non-EGFRvIII expressing tumours. Pharmacological inhibition of autophagy with the lysosomotropic drug chloroquine sensitizes EGFRvIII⁺ cells to hypoxia and nutrient deprivation. As we have shown before, reducing the hypoxic fraction by targeting autophagy with chloroquine treatment sensitizes xenografts to radiotherapy (17). This effect is more pronounced in EGFRvIII⁺ expressing tumours. In a retrospective study we showed that patients with EGFRvIII⁺ GBM benefit most from concurrent chloroquine treatment. These results suggest that EGFRvIII⁺ oncogene addiction in glioblastoma is a therapeutic vulnerability to chloroquine and could be a biomarker for patient stratification in clinical studies.

Strikingly, EGFRvIII⁺ cells consume more glucose suggesting that autophagy supports metabolic processes in EGFRvIII⁺ cells. The effects of autophagy inhibition on EGFRvIII positive cells, such as decreased hypoxia tolerance, can be rescued by pyruvate but not by glucose supplementation in the cell culture medium. This suggests that the glycolysis is already used to its fullest extent, and that autophagy is required as an additional en-

ergy source for these tumours. These data are in agreement with previous studies, showing that other mutations in the RAS/RAF/MEK/ERK pathway, such the BRAF^{V600E} mutation (1), but also HRAS^{G12V} or KRAS^{G12V} transformed cells show an increased dependency on autophagy (2).

Taken together, this study indicates that EGFRvIII⁺ have an increased metabolism and are more dependent on autophagy for their survival than EGFRvIII negative cells during metabolic stress. Remarkable results have been observed of EGFRvIII⁺ GBM patients treated concurrently with chloroquine and Radio- and chemotherapy (1). Currently 2 clinical trials (clinicaltrials.gov identifier NCT02378532 and NCT02432417) are initiated to evaluate toxicity of chloroquine in combination with radiotherapy and temozolamide, a chemotherapeutic commonly used for the treatment of GBM.

Autophagy degrades cyto- and genotoxic components, such as excessive ROS producing mitochondria, and is therefore considered a tumour suppressor mechanism. Several studies have demonstrated that defects in autophagy, due to deletions or mutations of proteins of the core autophagy machinery such as BECLIN1, can contribute to oncogenic transformation or at least neoplastic growth (18) (19,20). The ATG12-ATG5 complex acts as an E3-like ubiquitin ligase and functions in lipidation of LC3/GABARAP family proteins and is required for autophagy. In our study we observed that expression of the autophagy associated protein ATG12 is lost in ~40% of tumours in a cohort of head and neck cancer patients. Interestingly, patients with tumour with lost ATG12 expression show improved survival and tumour control, especially in stage 3 and 4.

Strikingly, patients with ATG12 negative tumours had almost no hypoxic fraction and responded better to therapies. It is common knowledge that autophagy inhibition sensitizes cells to internal and external stresses like hypoxia and nutrient deprivation, stressors which are common in the tumour micro environment. Therefore we hypothesized that defective autophagy (due to loss of ATG12 expression) sensitizes tumour cells to hypoxia leading to a decrease in tumour hypoxia, and since hypoxia is an important factor in therapy outcome, leading to improved therapy outcome. Indeed, in line with the observed effects in patients, xenografted ATG12 knockdown tumours do respond better to radiotherapy than their controls. However, this effect seems independent of the levels of hypoxia in these tumours as hypoxic fractions do not differ between control and knockdown groups. However, ATG12 deficient xenografts show a dramatically increase in tumour necrosis compared to control tumours, suggesting that these cells cannot survive in the tumour microenvironment due to stress exposure. Interestingly, ATG12 knockdown cells did not show an increased sensitization towards hypoxia alone, suggesting that a combination of stressors, such hypoxia and nutrient deprivation, is probably lethal. These combinations were not tested in the current study. The high fraction of ATG12 negative tumours prompted us to investigate if the loss of ATG12 expressing contributes to the oncogenic transformation of healthy cells. To test this we developed a model to specifically knockout ATG12 in the head and neck region and follow up tumour formation over time. This model was adapted from a previously de-

scribed study were 75% of mice with SMAD4^{-/-} in the head and neck had developed tumours in the oral cavity after 80 weeks (21). Therefore, SMAD4^{-/-} was taken as a positive control. In contrast to the original study, we did not observe excessive tumour growth in the head and neck area in any group, although we found an increase in anomalies in the ATG12^{-/-} group compared to controls characterized by larger thymus, salivary glands and tongue. Although these observations still need to be confirmed by pathological examination, these findings support the hypothesis that loss of ATG12 expression contributes to malignant transformation. Furthermore, ATG12 negative tumours are sensitized to stresses in the tumour microenvironment, probably due to defects in autophagy, what contributes to improved therapy outcome. Taken together, these findings imply the use of ATG12 as a biomarker to predict tumour hypoxia and treatment outcome. Tumour hypoxia is an important factor in treatment outcome, and is therefore important to determine. Currently, tumour hypoxia is not integrated in therapy treatments due to the lack of reliable detection methods.

Other endogenous markers of hypoxia, such as HIF and its downstream targets GLUT-1 and CA9 are dependent on hypoxia for their expression and do not always reflect the spatio-temporal dynamics of hypoxia and are therefore not suitable for clinical applications (22,23). In contrast to endogenous markers which aim for determining levels of hypoxia, ATG12 expressions seems to predict *if* a tumour is hypoxic, information which is relevant for treatment strategy.

Understanding mechanisms at play that allow tumour cells to survive hypoxia and adapt to the harsh environment is likely to result in future intervention possibilities. In this thesis we have addressed the complexity and versatile role of autophagy and autophagy-related proteins in tumorigenesis and tumour progression that based on our results may lead to novel prognostic tests and future treatment opportunities.

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Nederlandse samenvatting

Kanker is een van de belangrijkste doodsoorzaken in de westerse samenleving. Kanker ontstaat door de accumulatie van mutaties in het DNA. De belangrijkste oorzaak van deze mutaties is blootstelling aan schadelijke stoffen van buitenaf, maar kunnen ook worden doorgegeven door overerving. Wanneer een cel genoeg mutaties heeft verworven in essentiële genen kunnen deze cellen niet meer reageren zoals het hoort en kan dit leiden tot ongecontroleerde celdgroei waardoor een tumor ontstaat.

De groei van bloedvaten in een tumor draagt in belangrijke mate bij aan de ontwikkeling en groei van een tumor. Echter de vaatgroei in een tumor is in tegenstelling tot het vasculair systeem in gezond weefsel minder gestructureerd en meer chaotisch van aard. Mede hierdoor, en ook door de snelle groei van tumoren, komen er in een tumor gebieden voor die niet goed kunnen worden voorzien van zuurstof. Dit fenomeen heet hypoxie. Deze hypoxische gebieden zijn nadelig voor de prognose van een patiënt omdat deze gebieden in de tumor resistenter zijn tegen de meeste therapieën en dragen ze bij aan uitzaaiingen (metastasen). Omdat hypoxie elders in het lichaam niet voorkomt, liggen er ook kansen voor therapieën die specifiek zijn gericht op deze gebieden. Het werk in dit proefschrift beschrijft nieuwe bevindingen over hoe cellen zich gedragen tijdens hypoxie. Een van de mechanismen die cellen gebruiken om het gebrek aan zuurstof te overleven is autofagie. Autofagie is afgeleid uit het Grieks, en betekent zichzelf (auto) eten (fagie). Tijdens autofagie worden organellen en eiwitten afgebroken tot de essentiële bouwstenen zoals aminozuren, vetzuren en koolhydraten, waarna deze weer gebruikt kunnen worden voor de biogenese van onder andere nieuwe eiwitten die nodig zijn om de homeostase in de cel te bewaren. Autofagie is ook tijdens normale omstandigheden actief in de cel, maar wordt extra geactiveerd tijdens stress. Tijdens autofagie wordt het af te breken substraat ingepakt in een membraaneus blaasje, het autofagosoom. Deze autofagosomen fuseren met lysosomen die de enzymen bevatten om de inhoud af te breken tot de biologische bouwstenen. Het af te breken substraat wordt herkend door zogenaamde adaptereiwitten die het substraat koppelen aan het autofagosoom via eiwitten van de LC3/GABARAP familie, die direct in het membraan van onder andere autofagosomen zijn verankerd. Tot nu toe zijn er zeven eiwitten van de LC3/GABARAP familie beschreven: LC3A (2 splicingvarianten), LC3B, LC3C, GABARAP, GABARAPL1 en GABARAPL2. Deze eiwitten van de LC3/GABARAP familie delen een hoge mate van homologie, echter zijn ze allen uniek in hun functie. Een uitgebreid literatuuronderzoek is beschreven in **hoofdstuk 2** waar specifiek wordt ingaan op de individuele functies van elk van de eiwitten uit deze familie. Naast overeenkomsten, hebben LC3/GABARAP eiwitten unieke autofagie afhankelijke en onafhankelijke functies. Zo is de GABARAP familie onder meer betrokken bij het transport van receptoren van en naar het plasmamembraan. Zo hebben wij aangetoond dat hypoxie specifiek de expressie van GABARAPL1, en geen andere eiwitten uit de LC3/GABARAP familie, induceert. Tijdens deze condities is GABARAPL1 nodig voor het tot expressie brengen van epidermale groeifactor receptor (EGFR) op het plasmamembraan (**hoofdstuk 3**). De toegenomen expressie

van GABARAPL1 tijdens hypoxie wordt gereguleerd door de PERK-arm van de *Unfolded protein response* (UPR), een stressgereguleerde pathway die verkeerd- en ongevouwen eiwitten detecteert in het endoplasmatisch reticulum (ER) en daar op reageert.

Tumorhypoxie is een onafhankelijke prognostische indicator (marker) ongeacht de toegepaste therapie. Omdat GABARAPL1 verhoogd tot expressie komt tijdens hypoxie hebben we onderzocht of GABARAPL1 mRNA expressie gelinkt kan worden aan uitkomst van therapie. In een panel hoofd- halskankerpatiënten hebben we een correlatie aangetoond tussen GABARAPL1 expressie en verminderde tumorcontrole. Dit houdt in dat GABARAPL1 gebruikt zou kunnen worden als een biomarker om de uitkomst van therapie te voorspellen.

Epidermale groei factor receptor (EGFR) is een lid van de ErbB receptorfamilie en komt tot expressie in normale weefsels waar het is betrokken bij proliferatie en inhibitie van apoptose. Mutaties of overexpressie van deze receptor kunnen leiden tot verschillende aandoeningen zoals Alzheimer en kanker. EGFRvIII is een gemuteerde vorm van EGFR waarbij exon 2-7 zijn gedeleteerd en komt veelvuldig voor in glioblastoma multiforme (GBM). Doorgaans hebben GBM patiënten een zeer slechte prognose. Doordat het extracellulaire deel van de EGFR receptor is gedeleteerd, is de receptor constant actief waardoor groeisignalen continu worden doorgegeven, dat de groei van tumorcellen bevordert. EGFRvIII⁺ cellen zijn doorgaans resistent tegen hypoxie, wat resulteert in een grotere hypoxische fractie in een tumor. Verder groeien EGFRvIII⁺ tumoren doorgaans sneller en vindt er snellere hergroei van de tumor plaats na bestraling.

We hebben aangetoond dat tumoren met de EGFRvIII mutatie meer autofagie hebben en hiervan ook meer afhankelijk zijn voor hun overleving (**hoofdstuk 4**). Omdat hypoxie bijdraagt een verminderde prognose, hebben we geprobeerd de hypoxische fractie van een tumor te verlagen door autofagie te remmen met de drug chloroquine. Chloroquine remt autofagie waardoor cellen gevoeliger worden voor stress zoals nutriëntstarvatie en hypoxie. Verder hebben we aangetoond in muismodellen dat tumoren beter reageren op bestraling wanneer autofagie wordt geremd. Dit komt waarschijnlijk door het verlagen van de hypoxische fractie. Dit effect is het sterkst bij EGFRvIII⁺ tumoren. In een retrospectieve studie hebben we aangetoond dat GBM patiënten die gelijktijdig behandeld zijn met chloroquine meer voordeel halen uit de behandeling dan patiënten met EGFRvIII negatieve tumoren.

Autofagie breekt cytotoxische componenten af, zoals reactieve zuurstofverbinding-producerende (*reactive oxygen species* (ROS)) mitochondriën, en wordt daarom beschouwd als een tumorsuppressormechanisme. Verschillende studies hebben aangetoond dat autofagie-defecten, door bijvoorbeeld mutaties of deleties van eiwitten in de autofagiemachinerie zoals BECLIN1, bijdragen aan oncogene transformatie van cellen of minsten neoplastische groei. ATG12 is een essentieel eiwit van de autofagie machinerie en is betrokken bij de lipidatie van de LC3/GABARAP familie. Wij hebben aangetoond dat ATG12 expressie is verloren in ongeveer 40% van de tumoren in een cohort

van 428 hoofd-halskanker patiënten (**hoofdstuk 5**). Uit een ander cohort waarvan de hypoxieachtergrond bekend is, bleek dat ATG12 negatieve tumoren een sterk verlaagde hypoxische fractie hebben. Patiënten met een ATG12 negatieve tumor hebben daardoor een hogere kans op locale tumorcontrole en hogere overlevingskans in stadium 3 en 4 van de ziekte. Opvallend genoeg lijkt deze verlaging van de hypoxische fractie niet te zijn veroorzaakt door een defect in autofagie alleen. Doordat ATG12 ook een positieve regulator van apoptose is, gaan ATG12 negatieve cellen waarschijnlijk dood door andere mechanismen zoals necrose. Het niet naar behoren functioneren van autofagie en ongevoeligheid voor apoptose door het verlies van ATG12, leidt waarschijnlijk tot de vermeerderde necrose die we waarnemen in *in vivo* studies. Ook zou het zo kunnen zijn dat juist de combinatie van stressfactoren zoals hypoxie en nutriëntstarvatie zorgen voor celdood. Deze combinatie moet nog onderzocht worden.

Naast de rol in degradatie van cellulaire componenten, lijkt autofagie ook betrokken bij vormen van secretie. In **Hoofdstuk 6** hebben we uitgebreid literatuuronderzoek gedaan naar hoe autofagie-afhankelijke secretie bijdraagt aan tumorprogressie. Steeds meer onderzoeken laten zien dat autofagie betrokken is bij de secretie van tumorgroei stimulerende factoren, zoals groeifactoren maar ook immuunmodulerende factoren die bijdragen aan het immunosuppressieve karakter van het tumormicromilieu.

Tot deze factoren behoren ook de extracellulair vesicles (EVs). Dit zijn minuscule blaasjes die cellen onder andere gebruiken voor intercellulaire communicatie. Wij hebben aangetoond dat hypoxische cellen een subpopulatie EVs uitscheiden, gekenmerkt door GABARAPL1 expressie op het membraan (GABARAPL1⁺EVs) (**hoofdstuk7**). Deze EVs hebben pro-angiogenetische eigenschappen, dat wil zeggen dat ze de vorming van bloedvaten stimuleren dat weer bijdraagt aan tumorgroei. Tevens hebben we aangetoond dat deze GABARAPL1⁺EVs zijn te detecteren en verhoogd zijn in het bloed van kankerpatiënten. Doordat GABARAPL1 zich aan de buitenkant van deze EVs bevindt en in circulatie van de patiënt zijn, kunnen hier mogelijke therapieën op gericht worden. We hebben al aangetoond dat de functie van deze GABARAPL1⁺EVs geblokkeerd kan worden door middel van antilichamen die aan deze EVs binden. Momenteel zijn we bezig met de ontwikkeling van monoclonale antilichamen om de therapeutische werking te onderzoeken in muismodellen.

Naast pro-angiogenetische eigenschappen zijn GABARAPL1⁺EVs mogelijk betrokken bij de vorming van metastasen (**Hoofdstuk 8**). In metastaserende muismodellen zijn we in staat om het aantal longmetastasen met 80% te verminderen wanneer GABARAPL1 wordt uitgeschakeld door middel van een hairpin. Om te bewijzen dat het GABARAPL1⁺EVs zijn die een rol spelen bij de vorming van deze metastasen is nog verder onderzoek nodig. Wel zijn er aanwijzingen dat GABARAPL1⁺EVs een rol spelen bij de vorming van de zogenaamde “pre-metastatic niche”. Dat zijn gebiedjes elders in het lichaam die worden voorbereid op circulerende tumorcellen nog voordat deze hier kunnen nestelen en uitgroeien tot een metastase.

Valorisation

Despite great advanced in cancer research, cancer is one of the leading causes of death in western society. In solid tumours, areas deprived of oxygen (hypoxia) arise due to an aberrant and immature vasculature. Tumour hypoxia is a common characteristic of solid tumours and leads to expansion of tumour cells with a more invasive, metastatic and therapy resistant phenotype, and is therefore a major clinical challenge. The work in this thesis describes mechanisms that hypoxic cancer cells use to sustain their survival and thereby contribute to progression of the disease. Understanding of these mechanisms is important so that hypoxia can be detected and therapies can be directed to target these more resistant cells and improve treatment outcome. As a result of innovative research in the industry and academia, cancer survival rates have increased dramatically over the last decades and have a positive societal impact.

Clinical relevance

Tumour hypoxia is clinically important as hypoxic tumours show resistance to most cancer therapies and are therefore associated with decreased patient survival. Typically hypoxia reduces the effectiveness of radiotherapy. Depressed oxygen levels in tissue lead to a decrease of long-lived reactive oxygen radicals after X- or γ -irradiation that are required for fixed DNA damage of the tumour cell. Consequently, hypoxic cells are three times more resistant to irradiation than well oxygenized cells. Additionally, hypoxic cells are resistant to chemotherapeutic drugs. Typically, chemotherapeutic drugs target rapidly dividing cells. Hypoxia reduces cell proliferation and thereby limits the effects of chemotherapeutic agents. Also the decreased drug delivery due to the aberrant vasculature in the tumour contributes to resistance of chemotherapeutics due to delivery limitations. Also the propagation of metastasis and reduced the effectiveness of surgery are linked to hypoxia. Tumour metastasis is associated with high patient mortality and is responsible for more than 90% of cancer-related deaths.

Taken together, hypoxia is one of the strongest contributors to patient's poor treatment outcome. Therefore it is highly desired to develop methods to determine hypoxia in a tumour. Such a method can be used as a marker for treatment response but is also required to stratify patients for the right therapy plan and for the development of therapies targeting hypoxia.

Hypoxia cannot be determined anatomically, as hypoxia is independent of tumour size, grade and stage. Currently, there are no markers for tumour hypoxia used in the clinic for diagnostic or treatment purposes. Invasive polarographic electrodes are the 'gold standard' for assessing tumour hypoxia. However due to the heterogeneous and dynamic properties of hypoxia, multiple samples must be taken in the diseased tissue, and are due to the high burden for the patient never used. Determining hypoxia by imaging markers, such as HX4 scanning or others have not shown adequate specificity or do not correlate with hypoxia at all. Other described methods are the use of deter-

mining osteopontin in patient fluids. However due the low elevation of this marker in hypoxic tumours compared to healthy subjects, this method is of limited use when applied to a single patient. Taken together, methods to determine hypoxia in a tumour are limited and are not implemented in current treatment plans, but also to stratify patients for hypoxia-targeted therapies hypoxia markers are highly desired.

Improvement for healthcare

The work described in this thesis can be exploited to modify tumour hypoxia to improve therapy outcome, but also to develop targeted therapies and prognostic biomarkers.

In chapter 5, findings are described that ATG12 could serve as a predictor for tumour hypoxia and a tumour control in head and neck cancer patients. We found that in 25-40% of patients with head and neck cancer, tumours lack ATG12 expression. The loss of ATG12 expression correlates with a decreased hypoxic fraction in the tumour. Strikingly, patients with ATG12 'negative' tumours show improved local and loco-regional control. Therefore, ATG12 expression can be used as a hypoxia predictor and be implemented in pathohistological analysis after biopsies. Such a marker can contribute to stratify patients for clinical trials investigating hypoxia targeted therapies. I.e. ATG12 negative tumours are expected to be less sensitive to these therapies and should therefore be excluded from these trials. Because ATG12 expression is lost in a substantial group of head and neck cancer patients, institutes investigating the effectiveness of hypoxia targeting therapies in clinical trials should be aware of this phenomenon before including patients in their trials. To investigate the effectiveness of hypoxia targeted therapies, stratification of patients with hypoxic tumours is required. The finding that ATG12 negative tumours have almost no hypoxic fraction could contribute to improved outcome of these trials.

In chapter 7 we describe our findings that hypoxic cells secrete a subset of extracellular vesicles that is marked by GABARAPL1 expression on their surface. This finding provides means for both diagnostic and therapeutic applications and marketable products: Since GABARAPL1⁺EVs are released by hypoxic cells and are detectable in circulation of patients, GABARAPL1⁺EVs can be used as a hypoxia marker. We already showed that GABARAPL1⁺EVs are detectable and elevated in the blood of a small group (n=10) of colorectal, glioblastoma and small cell lung cancer patients. Currently our group is investigating if the number of circulating GABARAPL1⁺EVs correlate with the degree of hypoxia in a tumour to evaluate if we can determine the amount hypoxia. Enzyme-linked immunosorbent assays (ELISA) are suitable assays to determine macromolecular species in fluids and are frequently used as a diagnostic tool. Currently our group is developing ELISA assays to detect GABARAPL1⁺EVs in the blood of cancer patients. Such

a GABARAPL1⁺EV ELISA can be translated in a marketable product and could be exploited as such. A GABARAPL1⁺EV ELISA could provide information about the degree of hypoxia in a 'bench test' which is more desirable than invasive detection or expensive scanning methods which also require specialized personnel. Currently hypoxia activated pro-drugs (HAPs) are in development to specifically target hypoxic cells in a tumour. Tools to determine if a tumour is hypoxic are highly desired for patient stratification for these clinical trials.

The finding that GABARAPL1 is expressed on EVs also has therapeutic applications. Since GABARAPL1 is expressed on the surface of hypoxic EVs, it is accessible for targeting, for instance with antibodies. We already showed in chapter 8 that inhibition of GABARAPL1 with hairpin RNA's decreased metastasis formation by 80% in a metastatic breast cancer model in mice. Additionally, targeting GABARAPL1 resulted in decreased angiogenesis, tumour growth and tumour regrowth after irradiation and importantly, GABARAPL1⁺EVs are detectable and elevated in the circulation of cancer patients compared to healthy controls (chapter 7).

Therefore, we hypothesize that targeting GABARAPL1⁺EVs in the patient's circulation by GABARAPL1 targeting antibodies can reduce the formation of metastasis, but also might affect growth of the primary tumour; two important goals to achieve in patients. In chapter 7 we showed in a proof of concept experiment that we are able to block GABARAPL1⁺EV with antibodies targeting GABARAPL1 *in vitro* and thereby inhibiting their pro-angiogenic properties. Extrapolated to patients, this could include that this approach might prevent the formation of a pre-metastatic niche, and thereby *prevent* the development of metastasis.

These *in vitro* experiments were done with commercially available polyclonal antibodies. For therapeutic applications, typically monoclonal antibodies are used. Most used antibody subtypes are IgG1, 2 or 4, due to their long half-life in circulation (up to 21 days). We already have developed GABARAPL1 targeting monoclonal antibodies to further explore and validate this approach in *in vitro* and *in vivo* studies.

Since the use of the first monoclonal antibody in 1986, the market for these biopharmaceutical drugs has grown significantly. At present, 47 antibodies have been approved in the US or Europe. Annually, 4 new antibodies achieve marked approval, leading to an estimated world-wide sale of nearly \$125 billion in 2020 [1]. However, currently there are no therapeutics on the market or clinical trials investigating drugs targeting endogenous extracellular vesicles. In that perspective, this method could lead to the development of a novel and innovative anti-cancer therapy. The method to target EVs, and more specifically GABARAPL1⁺EVs, to prevent the formation of metastasis is described in a patent in Europe and the US (Keulers & Rouschop, patent number WO2015121295A1). To further develop this approach and translate this into a possible treatment we are looking for research partners to support further (co-)development and clinical proof of concept treatment. Such collaboration could lead to a university spin-off company to explore its clinical feasibility and efficacy.

GABARAPL1⁺EV targeting antibodies could be applied as a neoadjuvant therapy on patients with solid, and thus hypoxic, tumours. It needs to be empirically determined if GABARAPL1⁺EVs indeed are secreted by most solid tumours, but it is expected that most tumours release GABARAPL1⁺EVs. Most cancer related deaths are attributed the metastatic disease. Typically, distant metastases are frequently unresponsive to existing therapies and are treated systemically with palliative intent. Preventing the formation of metastasis could improve the life expectancy of many patients.

Tumour hypoxia contributes to poor treatment outcome in several cancer types. Therefore, reducing the hypoxic fraction in a tumour is highly desired. Attempts to modulate the hypoxic fraction were primarily focussed on increasing oxygen supply to the tumour. A different approach is alleviating hypoxia by targeting cellular mechanisms that use cells for their survival, such as autophagy.

Chloroquine is originally marketed as an anti-malaria drug, but is also used in the treatment of rheumatoid arthritis and lupus-associated arthritis. In addition to these properties, chloroquine is a potent autophagy inhibitor. Previously we showed that chloroquine sensitizes cells to hypoxia, reduces the hypoxic fraction and sensitizes xenograft tumours to radiotherapy in mice [2].

In chapter 4 we show that EGFRvIII expression elevates the activation of autophagy during hypoxia. The mutant form of EGFR, EGFRvIII is frequently observed in glioblastoma (50-60% of patients with EGFR amplification) and is associated with increased therapy resistance and poor prognosis. A retrospective study indicated that patients with EGFRvIII positive tumours benefit most of concurrent chloroquine treatment (median survival from 3 to 15 months).

Currently, a phase I clinical trial is ongoing in our institute (clinical trial NCT02378532) to determine the maximum tolerated dose of chloroquine with concurrent radiotherapy and temozolomid. So far, this trial shows promising results. To determine if chloroquine can be repurposed as a drug in oncology and considered as an additional treatment for GBM patients, additional clinical trials are required to investigate efficacy, effectiveness and safety.

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Tom

Curriculum Vitae



Tom Keulers was born on February 28th 1985 in Kerkrade, the Netherlands. In 2003 he obtained his secondary school degree at College Rolduc in Kerkrade. He then performed his bachelor studies (HLO) at Hogeschool Zuyd, Heerlen. In 2007 he obtained his bachelor's degree of applied sciences with a specialization in biochemical research. He continued his master studies Biopharmaceutical sciences at Leiden University, the Netherlands. During the first year of his master program he did an internship at the department of Biopharmacy, where he worked on apoptosis in atherosclerotic plaques. He continued his master program

in the group of Dr. Kasper Rouschop at the department of radiotherapy (Maastrro-Lab in Maastricht) where he laid the foundation for his PhD later in that group. After completing his master's degree with a specialization in drug-delivery technology and biopharmaceutics in 2012, he started as a PhD student in Dr. Rouschop's group. During this period he worked on the role of autophagy and extracellular vesicles in the hypoxic tumour microenvironment, which led to several publications and the discovery of GABARAPL1⁺EVs. This work was selected as 'late breaking abstract' at the international society for extracellular vesicles (ISEV), proffered presentations at international conferences, travel grants and was awarded with "best poster prize" at the European radiation research society (ERRS) 2016 and led to an international patent.

Recently Tom is granted *the Kootstra talent fellowship* to continue his research at the department of Radiotherapy at Maastricht University.

List of Publications

ATG12 expression predicts tumour hypoxia and tumour control in HNSCC

Tom G. Keulers, Alexander Koch, Hanneke Peeters, Kim G.M. Savelkoul, Nicole Bitsch, Roel G. Wanders, Dennis Roop, Johan Bussink, Veerle Melotte, Kasper M.A. Rouschop.
(In preparation)

Extracellular Vesicles as transmitters of hypoxia tolerance in solid cancer

Marijke I Zonneveld, **Tom GH Keulers** and Kasper MA Rouschop
(submitted)

GABARAPL1 is required for extracellular vesicle secretion during hypoxia

Tom G. Keulers, Sten Librechts, Marco Schaaf, Hanneke Peeters, Kim Savelkoul, Johan Bussink, Hans Duimel, Marc Vooijs, Barry Jutten, Marca Wauben, Kasper Rouschop
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EGFRvIII expression triggers a metabolic dependency and therapeutic vulnerability sensitive to autophagy inhibition.

Jutten B, **Keulers TG**, Peeters HJM, Schaaf MBE, Savelkoul KGM, Compter I, Clarijs R, Schijns OEMG, Ackermans L, Teernstra OPM, Zonneveld MI, Colaris RME, Dubois L, Vooijs MA, Bussink J, Sotelo J, Theys J, Lammering G, Rouschop KMA.
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Radiother Oncol. 2015 Mar;114(3):406-12. doi: 10.1016/j.radonc.2015.02.019

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Radiother Oncol. 2013 Sep;108(3):479-83. doi: 10.1016/j.radonc.2013.06.033

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Schaaf MB, Cojocari D, **Keulers TG**, Jutten B, Starmans MH, de Jong MC, Begg AC, Savelkoul KG, Bussink J, Vooijs M, Wouters BG, Rouschop KM.

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Patents

Method for determining the level of hypoxia in a tumor (EP3105593A1 & US20170176445A1).

K.M.A Rouschop & **T.G.H. Keulers**

*contributed equally

